ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

ion B MICROBIOLOGY AND IMMUNOLOGY

VOL 79B FASC 1-6 1971

UNKSGAARD COPENHAGEN

Munksgaard
International Booksellers and Publishers, Ltd
47, Prags Boulevard, DK-2300 Copenhagen S, Denmark



PRINTED IN DENMARK
VALD. PEDERSENS BOGTRYKKERI
COPENHAGEN

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VOL 79 B FASC 1-6 1971

TABLE 1 *Main Clinical Data on*

Patient	Sex	Age	Diagnosis*	Donor			Histo-compatibility group
				Sex	Age	Kinship	
T P	♀	42	CGN	♀	73	mother	D
P T	♂	23	CGN	♀	26	sister	C
H K	♂	37	CGN	♀	49	cadaver	C
S M	♀	15	CGN	♂	50	father	B
A S	♂	30	CGN	♀	56	mother	D
M L S	♀	22	CGN	♀	47	mother	C
S R	♀	49	NPreg	♀	13	cadaver	D
E L	♂	24	CIN?	♀	48	mother	D
E J	♀	13	CPyN	♀	43	mother	C
K L	♀	50	CPyN	♀	44	sister	A_1
L R	♀	50	CIN	♀	41	sister	C
A V	♂	10	Hered	♀	45	mother	D
A H	♂	44	Polycys	♀	48	sister	D

* CGN – Chronic glomerulonephritis CPyN = Chronic pyelonephritis CIN – Chronic interstitial nephritis NPreg – Nephropathy of pregnancy Hered = Hereditary nephropathy C_{CR} – Creatinine clearance

disease in one of them the diagnosis was uncertain This is indicated in the Table

Renal biopsies were studied 1 5–26 5 months after transplantation In all cases the biopsies were performed because of signs of deteriorating renal function or heavy proteinuria i e when rejection was suspected

The drugs prescribed at the time of biopsy were as follows all patients had prednisone 0 2–0 3 mg/kg/day all except H R and L R had azathioprine 1 0–2 0 mg/kg/day nine patients had combinations of diuretics, alpha methyldopa and dihydralazin as antihypertensive treatment Anti human lymphocyte globulin was not used

For control purposes four patients with chronic glomerulonephritis were studied Two normal kidneys removed at transplantation from cadaveric donors were studied as normal controls

Methods

In 12 cases the graft was biopsied percutaneously In one of them the graft was removed and reexamined In one patient only the removed graft was investigated Part of the tissue was fixed in 10 per cent neutral formaldehyde, embedded in paraffin wax, sectioned at 3–5 μ and stained with haematoxylin van Gieson, periodic acid Schiff and silver methenamine

Part of the renal *tissue specimen* was *snap* frozen and sectioned in a cryostat at 5 μ After drying overnight the sections were washed for 10 minutes in phosphate buffered saline (PBS) pH 7 2 Parallel sections were then incubated for 30 minutes with fluorescein labelled anti human immunoglobulin serum at room temperature in a humid atmosphere After incubation the slides were washed in two changes of PBS for 15 minutes and studied when still wet under a Wild fluorescence microscope (primary filter UG 1 (2 mm) secondary filter GG 13)

Five fluorescein labelled antisera were used to test immunofluorescence anti human immunoglobulin serum (AIg) anti human complement serum (AC) anti human IgA serum (AIgA) anti human fibrinogen serum (AFib) and anti human albumin serum (AAlb) The AIg serum was produced by immunizing a sheep with human IgG and IgM purified from serum as previously described (*Linder* & *Tallberg* 1969) The AC serum was produced by immunizing with zymosan adsorbed C 3 according to *Mardiney* & *Muller Eberhard* (1965) The AFib serum was produced by immunizing a rabbit with commercially available human fibrinogen (Kabi) The AIgA serum was produced by absorbing a polyvalent anti Ig serum reacting with IgG IgM and IgA with IgA deficient dysproteinaemic serum (*Tallberg* & *Jormalainen* 1968) The AAlb serum was produced by immunizing sheep with commercially available human albumin (Behringwerke AG)

Anti IgA serum (Sevac) was conjugated to serve as a control for the specificity of our anti IgA serum Indirect immunofluorescence using rab-

ths) ation	Renal symptoms at time of study: CCR (ml/min/1 73 m²)	Proteinuria (g/day)	Haematuria	Blood pressure at time of biopsy (mmHg)	Therapy: Steroids	Azathioprine	Anti-hypertensive drugs
	26	1 5	—	170/110	+	+	+
5	72	4 8	+	155/100	+	+	—
	18	9 0	+	190/110	+	+	+
5	38	2 5	—	155/100	+	+	+
	49	—	—	230/135	+	+	+
›	52	—	—	140/110	+	+	+
	11	7 8	—	230/130	+	—	+
	56	—	—	160/100	+	+	—
	74	6 3	—	155/110	+	+	+
	41	+	—	160/120	+	+	+
	27	—	—	160/90	+	—	—
	48	—	—	130/90	+	+	—
	5 9	5 0	+	190/120	+	+	+

anti IgA serum (Behringwerke AG) followed an antirabbit IgG conjugate (*Linder* 1969) was o performed as a control

The specificity of the antisera was tested by munodiffusion Antisera were conjugated with orescein isothiocyanate as described by *Linder* 969) The fluorescein/protein molar ration of conjugates varied from 1 5 to 2 5 Absence of nspecific reactions was confirmed using normal ney tissue as substrate The specificity of the ctions was tested by blocking the reactions by or incubation of sections with unconjugated iserum

RESULTS

ht Microscopy

With conventional staining the findings re fairly uniform Glomerular basement mbranes were thickened in twelve of the rteen biopsies The thickening was uneven, i was accompanied by proliferation of lothelial cells The proliferations were stly focal In two of the patients distinct ulation of the glomerular tuft was seen

In the two patients with the most ad nced glomerular changes about half of the meruli were hyalinized

The interstitial changes varied from slight oedema to heavy focal cellular infiltration with lymphocytes and plasma cells Occasionally eosinophilic granulocytes were seen

In some instances there were areas where the tubules were atrophic, contained casts and had thickened basement membranes

In five patients there were changes in arteries up to the size of interlobular arteries The changes consisted of thickening of the intima sometimes lamellar in character Arteriolar thickening was seen in these cases

Immunofluorescence

The results of the immunofluorescent staining is summarized in Table 2 One specimen had no glomeruli (A S)

Glomeruli When stained for IgG and IgM there was a weak linear fluorescence following the capillary loops in 7 biopsies (Fig 1) In one case (K L) there was a granular fluorescence No mesangial staining was seen The AIgA serum stained the glomeruli in a different way, usually producing a granular fluorescence along the ca-

TABLE 2 *Light and Immunoflu*

Patient	Glomeruli						Light
	GBM thickening	Lobulation	Hyalinization	Immunofluorescence			microscop (atrophy)
				IgG IgM	C	Fib	
T P	+	—	+	±	+	—	+
P T	+	—	—	±	+	—	—
H K	+	—	+	+	+	/	+
S M	—	+	+	+	+	—	+
A S	+	—	—	/	/	/	—
M L S	+	—	—	±	—	—	—
S R	+	—	—	±	+	—	+
E L	+	—	—	+	—	—	—
E J	+	+	+	—	—	—	+
K L	+	—	+	+	+	+	—
I R	+	—	—	+	+	/	—
A V	+	—	—	+	+	+	—
A H	+	—	+	+	+	+	+

+ = Positive finding ± = Insignificant finding — = Negative finding / = Not studied

pillary loops and Bowman's capsules The fluorescence was often patchy in the hilar region, and in some glomeruli a lumpy dense fluorescence was seen in the capillary loops In some capillary loops the fluorescence appeared almost linear Staining with AC' serum gave similar results to those with AIgA serum (Fig 2) Staining for fibrinogen produced a granular or linear fluorescence, usually only partly affecting the glomerulus (Fig 3) The fluorescence was weaker than that produced by AC' and AIgA sera, and did not affect all the glomeruli in each specimen Fibrinogen was found in three cases (K L, A V, A H) No fluorescence was seen when sections were stained for albumin

Tubules Sections stained for IgA or C'3 characteristically contained tubules with highly fluorescent basement membranes The fluorescence was patchy or granular and often interrupted (Fig 4) In the cortical region almost all the tubules were stained, while the medulla usually lacked fluorescence In one biopsy AIgA serum stained the cytoplasm as well as the basement membrane (H K) In another specimen (A V) only the cytoplasm stained with AIgA, but the basement membranes stained for complement The tubules were not stained by antibodies against IgG and IgM or against fibrinogen All antisera, except AFib stained tubular casts

Vessels In six cases vessels of all sizes reacted with both AIgA and AC' sera (Fig 5) Staining for IgG IgM was positive in four cases (H K, K L, A V, A H) Usually both the thickened intima and the media were affected The fluorescence was patchy and sometimes formed concentric strands in cross sections (Figs 6 and 7) In some arterioles the lumen was almost obliterated by the homogenously stained thickened intima (Fig 6) In some cases (S R, A V, A H) AFib produced a spotted intertubular fluorescence This was probably due to staining of intertubular capillaries

In sections from four glomerulonephritic kidneys stained for immunoglobulins, complement fibrinogen and albumin the fluorescence could not be distinguished qualitatively from that in the kidney transplant biopsies IgA was not present in one case Quantita

ings on 13 Renal Biopsies

unofluorescence		Vessels			
		Light microscopy (thickening)	Immunofluorescence		
C	Fib		IgG IgM	C′	Fib
+	—	+	—	+	—
+	—	—	—	+	—
+	/	+	+	+	/
+	—	+	—	+	±
+	/	+	—	—	/
+	/	—	—	—	/
+	—	—	—	+	+
—	—	—	—	—	—
+	/	—	—	+	/
+	—	—	+	+	±
+	/	—	/	/	/
+	—	—	+	+	+
+	—	+	+	+	+



ig 1 Glomerulus showing linear fluorescence in capillary basement membranes Granular fluorescence
ı afferent arteriole (lower right) and subintimal fluorescence in vessel wall (left) Cryostat section
ained with fluorescein conjugated antiserum against human IgG and IgM Original magnification
00 ×

tive variations were however seen with the AIgA and AC′ sera In the glomerulonephritic kidneys the fluorescencent material was more granular and often seemed to occupy the lumen of glomerular capillaries (Fig 8) Mesangial fluorescence was also noted

Immunofluorescence controls for the specificity of the reactions with anti IgA serum was tested in one case (S R) in which the rejected kidney had been removed The control anti IgA sera failed to produce the typical staining pattern obtained with our anti IgA serum, but gave a cytoplasmic fluorescence in some tubules As the localization of this antiserum was very similar to that obtained with the AC′ serum the possible contamination of the AIgA serum with antibodies against C′3 was tested In immunodiffusion the AIgA serum produced no deviation of the precipitin line produced by AC′ serum reacting with normal human serum An attempt to block the reaction obtained with AIgA serum by incubation of sections with unconjugated AC′ serum prior to incubation with AIgA also failed

No staining was observed in the normal kidneys using the different conjugates

DISCUSSION

This series of thirteen renal transplantations contains recipients with renal diseases of different aetiology In glomerulonephritis immunological mechanisms are involved in the pathogenesis and the original disease might be transferred to the graft In the other recipients with chronic pyelonephritis chronic interstitial nephritis or urogenital anomalies the original disease is not likely to recur All patients had clinical signs of deteriorating renal function after transplantation There were no clinical features that would have justified a separation of the patients into a group with recurrent glomerulonephritis and a group with chronic allograft reaction

With the light microscope we almost always found thickening of glomerular basement membranes focal endothelial proliferation and lobulation of the glomeruli together with interstitial and vascular changes These have been reported frequently in allografted kidneys (*Porter et al* 1967, *Kincaid Smith* 1967) The light microscope findings do not permit any definite conclusions to be drawn about the aetiology of the glomerular changes

Several immunohistochemical studies (*Porter et al* 1968, *McKenzie* & *Whittingham* 1968 *Jonasson et al* 1967) have showed deposits of IgG IgM and complement in the glomeruli and vessels of both healthy renal allografts and those being rejected Both linear and granular deposits in the glomerular basement membrane have been described The findings have thus been suggestive of the two forms of glomerulonephritis the one with linear deposits caused by anti glomerular basement membrane antibodies and the other with granular deposits caused by circulating antigen antibody complexes (*Dixon* 1968) These patterns of immunoglobulin deposition have been reported even in cases where there was no glomerulonephritis before renal transplantation (*Porter et al* 1968) It is therefore highly probable that both circulating antigen antibody complexes and anti glomerular basement membrane antibodies operate and produce the changes seen in chronic rejection of renal transplants

In our series some characteristics of the immunohistochemical staining pattern seem to be important

A clearcut division of the immunofluorescence staining into linear or granular was often impossible The basement membranes

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Fig 2 Glomerulus showing deposition of complement The fluorescence in capillary basement membranes almost linear Granular fluorescence dominating in the hilar region Note massive staining of afferent arteriole Cryostat section Original magnification 200 ×

Fig 3 Glomerulus showing deposits of fibrinogen Scattered lumpy fluorescence notably in the hilar region is seen Cryostat section Original magnification 200 ×

of the tubules often had granular staining for IgA and complement, while those of the glomeruli had both linear and granular staining for immunoglobulins and complement It therefore seems possible that both circulating complexes and antibodies reacting with the glomerular basement membrane play a role in the same patient with a renal graft The relative uniformity of the immunohistochemical pattern in our series implies that the original disease of the recipient does not affect this finding There were no results that would have justified a separation of the patients into groups with and without glomerulonephritis

Quantitative differences in antibody activity against immunoglobulins complement and fibrinogen may be one reason why our preparations frequently stained with anti IgA while antisera against IgG IgM or fibrinogen gave weak or negative reactions in glomeruli and vessels

It is difficult to interpret the possible significance of the observed reactions using our AIgA serum Although there was no doubt regarding the monospecific activity of this antiserum in immunodiffusion tests the failure of the anti IgA sera used as controls to produce a similar result makes us doubt that the observed fluorescence was not due to a reaction with IgA in the sections As the material used for immunization consisted of antibodies eluted from immunoadsorbents it is possible that complement factors other than C 3 were present in sufficient amounts to produce antibodies upon immunization The failure to demonstrate any contaminants by immunodiffusion may be due to the absence or presence of only minute amounts of these factors in normal sera

The frequent finding in our series of deposits of C'3 especially in the tubular basement membranes differs from what has been reported before

The presence of C'3 in the basement membranes of the tubules suggest that complexes have formed in the tubules or at their basement membranes If an antigen of the tubules is involved it may be derived from the basement membranes or from the cells It is also possible that it is adsorbed from the glomerular filtrate and that it is the glomerular basement membrane antigen that is normally found in urine and blood and which is involved in the production of glomerulonephritis (*Lerner et al* 1967 *McPhaul & Dixon* 1969)

The immunohistochemical staining of the vessels was similar to that reported previously (*McKenzie & Whittingham* 1968) Vessels of all sizes were affected very uniformly There are two possible explanations of these lesions They could be due to the allograft reaction per se or they could be related to the arterial hypertension present in most of the cases It seems unlikely that arterial hypertension is the main factor, because the grafts of non hypertensive patients had similar vascular lesions Although the vascular lesions could be due to the allograft reaction they were also found in the kidneys of our glomerulonephritic patients

It is possible that there is a difference in the immunohistochemical staining properties of different grafts depending on factors such as histocompatibility, time after transplantation and severity of allograft reaction Our series was characterized by a high proportion of cases with poor histocompatibility and clinical rank

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We thank the following members of the transplantation team Drs *Anja Tiilikainen* M D *B Kuhlback* M D and *B Lindstrom* M D for their contribution

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Fig 4 Longitudinal section of proximal secreting tubules in cortical region Staining for IgA Granular fluorescence in tubule basement membranes In the cytoplasm of some tubule epithelial cells there is a weak fluorescence Cryostat section Original magnification 200 ×

Fig 5 Section from cortical region of removed transplant showing a bifurcating interlobular artery Intense staining for complement in the vessel media Staining of tubule and glomerular basement membranes is also seen Cryostat section Original magnification 35 ×





7,

s 6 and 7 Intense fluorescence in interlobular ry stained for complement Lumpy fluorescence media of vessels In Fig 6 there is an additional nogeneous subintimal deposit In Fig 7 the nching point of an arteriole is seen Cryostat tion Original magnification 200 ×

8 Typical staining of glomerulus from kidney cted by chronic glomerulonephritis Section ned for complement Lumpy fluorescent ma al often occupying the lumen of glomerular illary loops is seen Note homogeneous staining the wall of the afferent arteriole seen in cross tion to the left Cryostat section Original mag- cation 200 ×

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LIPOPOLYSACCHARIDE FROM *BACTEROIDES MELANINOGENICUS* ISOLATED FROM THE SUPERNATANT FLUID AFTER ULTRACENTRIFUGATION OF THE WATER PHASE FOLLOWING PHENOL-WATER EXTRACTION

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Lipopolysaccharide (LPS) has been isolated from the supernatant fluid after ultracentrifugation of the water phase following phenol water extraction of *Bacteroides melaninogenicus*, strain B10 The purification procedure included digestion of the supernatant fluid with ribonuclease and deoxyribonuclease gel filtration on Bio Gel A 1 5 m and ion exchange chromatography on DEAE cellulose The prepared LPS did not contain heptose or 2 keto-3 deoxyoctulosonic acid The sugar components identified were galactose glucose mannose, rhamnose traces of fucose, galactosamine and glucosamine Neutral sugar and lipid constituted the main parts of the LPS The isolated LPS sensitized sheep erythrocytes to agglutination in homologous antiserum Two agar precipitation lines were observed one of which may correspond to an acid hapten

The chemical composition and the serological and biological activities of lipopolysaccharide (LPS) from *Bacteroides melaninogenicus* have been reported (4, 5, 6) LPS was extracted from whole cells with phenol water and purified by precipitation in the ultracentrifuge Further chemical examinations of the LPS preparations showed however, that they were heavily contaminated with glycogen

Attempts to purify the contaminated LPS as well as attempts to avoid the contamination by modifying the extraction procedure and by using isolated walls for extraction have been unsuccessful Glycogen free preparations have now been prepared from the supernatant after ultracentrifugation of the water phase following phenol water extraction The present paper deals with the purification procedure and the chemical composition of the LPS obtained Some sero-

Received 15 vii 70

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ical properties of the preparations are
) described

METHODS

teroides melaninogenicus strain B10 (4) was
tivated in nutrient broth enriched with 0 5 per
t glucose 0 3 per cent yeast extract, 0 1 per
t cysteine 0 0005 per cent haemine, 0 0001 per
t menadione and 4 per cent horse serum The
of the medium was adjusted to 7 0

Extraction and purification methods Extraction
acetone-dried whole cells with phenol water
3) and fractionation of the water phase by ultra
ntrifugation (100 000 *g* for 1 hour) was carried
t as described earlier (4) The washed pellet
tained by ultracentrifugation is referred to as
S-sed, and LPS prepared from the super
tant fluid as LPS sup Gel filtration was per
ed on columns of Bio-Gel A 1 5 m 100–200
th agarose content 8 per cent (Bio-Rad Labo-
ories Richmond, California USA) The col
ns were stabilized and eluted with 0 1 M tris
ydroxymethyl) aminometan buffer pH 7 8 con
ning 0 001 M EDTA and 0 02 per cent sodium
de The void volume of the gel filtration col
ns was measured in separate runs in which
ue dextran was passed through with the same
ffer Columns for ion exchange chromatography
re prepared from DEAE-cellulose (DEAE SS,
rva Heidelberg Germany) Elution was per
med with 0 02 M phosphate buffer pH 7 4 and
th a NaCl gradient in the same buffer Digestion
th ribonuclease (5 × cryst, ex bovine pan
as Sigma Chemical Company St Louis Mis
ri USA) and deoxyribonuclease (25 per cent
tivity of crystalline material deoxyribonuclease
bovine pancreas L Light & Co Ltd, Coln
ook England) was carried out in 0 1 M phos
ate buffer, pH 7 0

Paper chromatography Acid hydrolysis was per
med in sealed tubes at 100° C with 3 or 4 N HCl
r 3 or 4 hours respectively or with N H_2SO_4
r 10 min 4 or 16 hours as required Acid was
moved from HCl hydrolysates by evaporation
er NaOH pellets The H_2SO_4 hydrolysates were
utral zed by passage through a column of Do
x 1 in the formate form Sugars were separated
circular paper chromatography with *n* butanol
ridine water (6 4 3) and stained by silver ni
te or aniline hydrogen phthalate Amino sugars
re also detected with the Elson Morgan reagent
2)

Chemical analyses Neutral sugars were measured
the Winzler orcinol method (19) with glucose
lactose (1 1) as standard and methyl pentoses
the sulphuric acid-cysteine reaction (2) Rham
se was used as standard Hexosamines were
timated as glucosamine HCl (14) Samples were
hydrolysed at 100° C with 3 N HCl for 4 hours
Nitrogen was determined by the micro-Kjeldahl
method (8), protein by the Folin Ciocalteau phenol
method (10) with bovine serum albumin as
standard Fatty acid esters were determined as
tripalmitin by the hydroxamic acid method (15)
The presence of 2 keto-3-deoxy-octulosonic acid
(KDO) was investigated by the malonealdehyde-
thiobarbituric acid method (17), in samples hydro
lysed in 0 02 N H_2SO_4 for 20 min at 100° C
Heptose was sought as described by *Dische* (1)

Serological methods Ring test precipitation and
double diffusion in agar were performed as de
scribed earlier (5) Immunoelectrophoresis was
carried out with equipment type LKB 3276 B
(LKB Produkter AB, Stockholm, Sweden) in 1 per
cent agar gel with veronal buffer, pH 8 6, ionic
strength 0 05 Electrophoresis was run for 1 hour
at 250 V After application of antiserum the plates
were incubated at room temperature for 1 or 2
days Sensitization of sheep erythrocytes for indi
rect haemagglutination was performed as described
previously (5), with untreated LPS or LPS treated
with 0 25 N NaOH for 1 hour at 56° C The
haemagglutination tests were performed using the
Microtitre equipment (Flow Laboratories, Irvine
Scotland) with 0 025 ml of serum dilution and
0 025 ml of a suspension of 0 5 per cent sensitized
sheep erythrocytes Otherwise the tests were per
formed as described (5) For inhibition of haem
agglutination 0 025 ml of diluted antiserum con
taining eight agglutinating doses were pre incu
bated at 37° C for 1 hour with inhibitor in 0 025
ml buffered saline Antiserum against LPS pre
cipitated by ultracentrifugation (antiserum LPS
sed) was prepared as described (5)

RESULTS

Purification of supernatant LPS (LPS sup)
The supernatant after ultracentrifugation of
the water phase from phenol water extrac
tion of *B melaninogenicus* contained con
siderable amounts of material with the same
serological specificity on agar precipitation
as the LPS sed (Fig 3), and agglutination
of sheep erythrocytes sensitized with LPS
sed by antiserum LPS sed was completely
inhibited by freeze dried supernatant Fur
thermore, the supernatant fluid produced a
positive epinephrine skin test (16) in rabbits

The supernatant contained relatively large
amounts of nucleic acid components as indi
cated by a peak of absorption at 260 mμ The
bulk of these contaminants could be sepa
rated from the active material by gel filtra

TABLE 1 *Percentage Chemical Composition of Lipopolysaccharide from* Bacteroides melaninogenicus *B10, Isolated from the Supernatant Fluid after Ultracentrifugation of the Water Phase following Phenol Water Extraction of Acetone Dried Whole Cells*

	Neutral sugar	Methyl pentose	Hexosamine	Fatty acid ester	Nitrogen	Protein
Batch 1	26 8	6 4	11 9	25 5	3 1	4 7
Batch 2	33 0	9 1	8 5	30 8	3 7	4 2

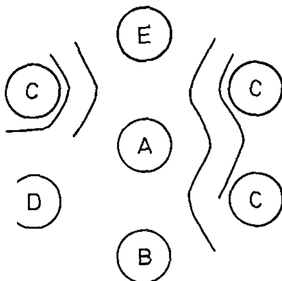


Fig 3 Schematic drawing of agar precipitation lines produced by purified LPS sup 0 2 mg/ml (well A), LPS sed 5 mg/ml (well B), LPS sed hydrolysed at 100° C in 0 1 N acetic acid for 1 hr 2 mg/ml (well D) and lyophilized crude super natant fluid after centrifugation 1 mg/ml (well E) against antiserum LPS sed (well C)

was visible with suspensions containing less than 0 5 mg/ml of hydrolysed LPS sed (Fig 3)

The minimal concentrations of LPS sup required for sensitization of an equal volume of a 0 5 per cent suspension of erythrocytes for haemagglutination was 25 µg/ml, irre spective of previous treatment with sodium hydroxide The antibody neutralizing capa city of LPS-sup was measured by inhibition of agglutination of erythrocytes sensitized with untreated or alkali treated LPS sup, using eight agglutinating units of antiserum Minimal inhibitory doses of untreated and alkali treated LPS sup are shown in Table 2

TABLE 2 *Minimal Doses of Untreated and Alkali Treated LPS sup Required for Inhibition of Ag glutination of Sheep Erythrocytes Sensitized with Corresponding Antigens, in Eight Agglutinating Units of Antiserum LPS sed*

Antigen used for sensitization	Minimal inhibitory dose, µg	
	Untreated LPS sup	Alkali treated LPS sup
Untreated LPS sup	0 01	3 0
Alkali treated LPS sup	0 005	0 0025

DISCUSSION

Approximately 75 per cent of the ring test active material in the freeze-dried super-natant fluid after ultracentrifugation of the water phase following phenol water extrac-tion of *B melaninogenicus* strain B10 was recovered in the LPS-sup The degree of purification, also calculated from dry weight and ring test activity, was about 80 fold

The elution pattern on DEAE cellulose indicates heterogeneity of the LPS sup pre-parations LPS sed shows the same hetero geneity (4) which appears to be a common observation with endotoxic bacterial poly-saccharides (11)

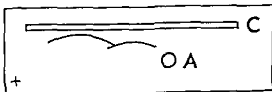


Fig 4 Immunoelectrophoresis of LPS-sup 1 mg/ ml (A) against antiserum LPS sed (C)

cause of the limitations of the methods d and the low recovery, the quantitative lyses performed can only give a rough mate of the quantitative chemical com ition of LPS sup The values obtained for osamine and protein do not account for the nitrogen in the preparations Incom te hydrolysis may have given too low ues for hexosamine in the Elson Morgan ction (3) It is also possible that LPS sup tains more polypeptide than reflected by Folin Ciocalteau reaction which is de ied for soluble whole proteins, and in ich the colour intensity may vary from protein to another

The LPS-sup preparations from *B me inogenicus* contain the same sugar com ients as do LPS from *B fragilis* NCTC 3 extracted in the same way, but purified sedimentation in the ultracentrifuge (7) ptose and KDO are lacking in both LPS o the quantitative chemical composition ery similar

ralactose mannose and fucose were not ected in the glycogen contaminated LPS preparations from *B melaninogenicus* viously examined (4) Acid hydrolysates everal LPS sed preparations have there been re-examined by paper chromato phy Trace amounts of galactose, mannose fucose were found when extraordinary e amounts of the hydrolysed samples were lied to the paper

n haemagglutination inhibition experi its the minimal inhibitory doses of un ted LPS sup were relatively low regard of whether the LPS sup used for sensi tion of erythrocytes had been treated with li or not Alkali treated LPS-sup at low centrations also inhibited the aggluntina of erythrocytes sensitized with similarly ted LPS sup When on the other hand eated LPS sup had been used for sen ation alkali treated LPS sup was much effective as inhibitor These findings may est the presence in LPS sup of several genic determinants the nature of which ains to be examined

he results of agar precipitation experi ments with acid hydrolysates of LPS-sed strongly suggest a relationship between the two precipitinogens in LPS-sup from *B melaninogenicus* strain B10 similar to that observed in endotoxin preparations from *Enterobacteriaceae* by *Ribi et al* (13) and in *Fusobacterium* by *Kristoffersen* (9) This would imply that the rapidly diffusing precipitinogen corresponds to the so-called acid hapten The difference in electrophoretic mobility is currently being exploited as a possible means to separate the two pre cipitinogens

The results of the serological investigations reported previously (5) and in this paper show that LPS from *B melaninogenicus* B10, in spite of having a core lacking heptose and KDO exhibit serological properties analogous to typical LPS prepared from *Enterobacteriaceae* and other Gram negative ba cilli

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IMPROVED TECHNIQUE FOR SEPARATION AND ANALYSIS OF SOLUBLE IMMUNE COMPLEXES

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An improved technique for separation and analysis of soluble *immune complexes* by rate-zonal ultracentrifugation is described The separation is achieved with a B XIV titanium zonal rotor and a 3 to 23 per cent (w/w) optimized isokinetic sucrose gradient This method allows an accurate calculation of sedimentation coefficients for separated complexes

he study of the reaction between antigen antibody, we have previously used rate l ultracentrifugation for separation and ysis of soluble immune complexes formed een iodo-acetamide alkylated [131]I labe d human serum albumin *monomer* SA-mon) and rabbit anti human serum min (Ab) (1) For these experiments ised a B-XIV aluminium zonal rotor (2) an isokinetic sucrose gradient, found rically However, working with a B-XIV ium zonal rotor (2) and a computer tructed isokinetic sucrose gradient (3), possible to obtain a far better resolution ie immune complexes, as reported in the nt paper The high power of resolution n isokinetic sucrose gradient is demon ed by comparison with a linear (on ne) sucrose gradient In addition, it is n that the shape of a zone closely reles the Gaussian frequency function

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quests for reprints should be addressed to *ensgaard*, Biokemisk Institut, Aarhus Uni et DK 8000 Aarhus C, Denmark

MATERIALS AND METHODS

Alkylated [131]I labelled human serum albumin monomer (HSA-mon), rabbit anti human serum albumin immunoglobulin (Ab), and the soluble immune complexes were prepared as described previously (1) [131]I activity was determined in a Packard Autogamma spectrometer

A Superspeed 65 Mk II ultracentrifuge (*Measuring and Scientific Equipment Co Ltd London* SW 1) equipped with an electronic force-time *integrator*, a B XIV titanium zonal rotor, and an MSE *automatic* variable gradient former was used The volume of sample (*containing approx* 1 mg of total protein) was 2 ml, and of *overlay* 100 ml (tris buffer, 0 05 M pH 8 0) The *rotor* temperature during the run was 8° C, controlled by an infra red radiation thermostat Gradient and overlay were pumped into the rotor at 6° C to *compensate* for heating of the fluids by passage through the feed head 700 ml of a 28 per cent (w/w) sucrose solution was used as cushion The computer constructed isokinetic *sucrose* gradient (3) is shown in Fig 1 The stock solutions consisted of 2 99 per cent and 22 86 per cent (w/w) *sucrose* in 0 05 M tris buffer pH 8 0 The gradient was produced in the gradient former by *mixing* the stock solutions in ratios stated on a template having percentage of the light solution as the ordinate and percentage of the total gradient volume as abscissa The centrifugation was con tinued at about 46000 *rev/min* until a pre-

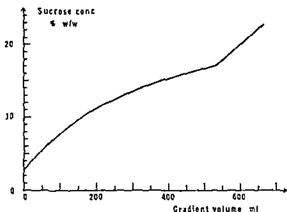


Fig 1 Profile of the isokinetic sucrose gradient for separation of particles with $s_{20\,w}$ values between 0 and 35 S

determined force time integral (4 54 × 10¹¹ rad² sec) was achieved, corresponding to a running time of approx 300 min and an acceleration and deceleration period of about 15 and 10 min (with brakes on), respectively The cut volume was about 9 4 ml

The sucrose concentration in each cut was determined by measurements of the refractive index at 20° C, using a Bellingham and Stanley high accuracy Abbe '60' refractometer with a thermostated prism housing and a Leybold Sodium Lamp The refractometer readings were converted to sucrose concentration by means of a Gier Algol 4 program, based on standard tables (4)

Sedimentation coefficients for all cuts were calculated using a Gier Algol 4 program based on *Bishop* (5) and *Barbers's* (6) theoretical considerations The sedimentation coefficient, corrected to the standard state ($s_{20\,w}$), water at 20° C, is formally defined by the equation

$$s_{20\,w} = \frac{1}{\omega^2 t} \times \frac{\rho_p - \rho_{20\,w}}{\eta_{20\,w}} \times \int_{r_0}^{r_t} \frac{\eta_{T\,m}}{\rho_p - \rho_{T\,m}} \times \frac{dr}{r}$$

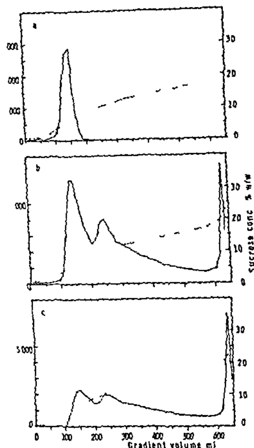
where $\omega^2 t$ is the force time integral ρ the density, η the viscosity and r radius in the rotor The indices $_p$, $_T$ and $_m$ refer to the particle, the temperature and a *sucrose containing* medium respectively r_0 is the sample band *position and* r_t *the radius* for a given fraction For each fraction the density and viscosity is calculated from the measured sucrose concentration by means of a set of polynomial expressions given by *Barber* (6) The radius for a given fraction is found by linear interpolation in a table containing paired *values of* radius and volume (3) The sedimentation coefficient for each fraction was then obtained by trapezoid integration The particle density was taken as 1 39 (1)

The shape of the zone in Fig 2a has been investigated by comparison with a Gaussian curve by means of a previously described program for Kolmogorov Smirnov statistics (7) In the diagram (cf Fig 4) the ordinate is the ordinate to the normal cumulative distribution function, and the abscissa is $(x - \bar{x})/s$ where x is observed value, $\bar{x}$ is the average and s the estimated standard deviation of population The smooth curve is the normal cumulative distribution function The empirical distribution function is drawn as a step function with each step representing one cut.

RESULTS AND DISCUSSION

Fig 2 shows the results of rate zonal ultracentrifugation of a) *HSA mon, b) and c) immune complexes formed in moderate and low antigen excess If the antigen/antibody ratio at the point of maximum precipitation on the titration curve is set to 1 1, the antigen/antibody ratio in b) and c) were 3 1 and 2 1, respectively In Fig 2 a) one distinct peak is seen with a $S_{20\,w}$ value of 4 8 which closely corresponds to the sedimentation coefficient of pure human serum albumin (8) In b) and c) two peaks having sedimentation coefficients of about 4 8 and 12 7 S respectively are seen These two peaks contain free HSA and soluble immune complexes, respectively The shape of the trailing side of the 13 S peak in b) and c) indicates the existence of soluble immune complexes with sedimentation coefficients up to 35 S Thus, the elution pattern of the immune complexes, separated by zonal centrifugation, presents a picture, which is in full agreement with analytical ultracentrifugations of immune complexes (9)

We have compared the present technique with the technique described previously (Fig 2b) and 3a)) As can be seen, the peaks are more narrow and the separation is better There are several reasons for the improvements obtained Using 2 ml 0 05 per cent *HSA mon as sample the initial width of the zones, as measured by the width of the peak at half of its height by unloading immediately



2 Zonal centrifugation with the improved hnique of a) antigen alone and b) and c) mix es of soluble immune complexes and antigen ess The solid curves (left ordinates) represent radioactivity The broken curves (right ordi es) show the *sucrose concentration*

er having completed the loading includ overlay was 14 8 ml using 100 ml over against 18 9 ml using 250 ml overlay rthermore the higher maximum speed of e titanium rotor permits the use of a eper gradient which in turn causes less oadening of the zones during the centri gation (10) After centrifugation of 2 ml ISA mon by the present technique we und a zone width of 40 ml against 50 ml th the previously described technique (the perimental conditions are stated in legend Fig 3a))

The two techniques are comparable in this ty as the gradients in both cases are con structed to achieve a uniform distribution of 4—to 35 S particles over as large a volume in the rotor as possible, by use of a five hour run at maximum speed

The improved separation of the 4 8 and the 13 S peaks is due to the larger effective radius in the rotor (32 mm against 20 mm) when using 100 ml overlay instead of 250 ml

Fig 2c) and 3b give a comparison of two identical samples separated on an isokinetic and a linear (on volume) gradient, respectively As seen, the separation on the isokinetic gradient is better, as the peaks are narrower than by use of the linear gradient

Knowledge of the shape of the zones in the rotor is important as it can be used to detect overloading of the gradient (10), and can provide the basis for resolving a complex elution pattern into individual peaks Although a zone, in a mathematical sense, is not a genuine frequency function it appears from Fig 4 that the shape of the integrated

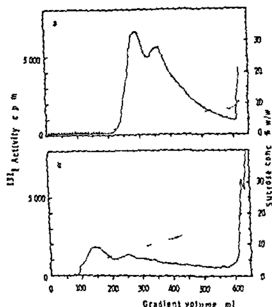


Fig 3 Zonal centrifugation of soluble immune complexes with a) the previously described technique (1) and b) with the present technique but using a linear gradient In a) a B-XIV aluminium rotor 250 ml overlay 2 ml sample, a 5 hour run at *30000* rev /min and 8° C was used The force *time integral* was 1 84 × 10[11] rad[2] sec

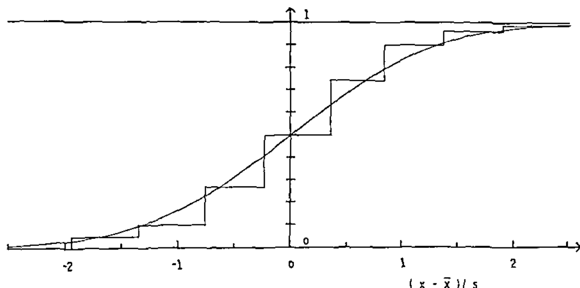


Fig 4 Comparison between the normal cumulative distribution function (smooth curve) and the integrated shape of the zone, (the step function) For further explanation see Materials and Methods

zone curve closely resembles the shape of the Gaussian distribution function The use of this approach provides the advantage of permitting, in contrast to a Gaussian test, the inclusion of the leading and the trailing edges of a zone in the test

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The authors wish to thank Mrs *Kirsten Wigard* and Mrs *Lis Claudi* for their technical assistance This work was supported in part by a fund from *F L Schmidt and Co's Jubilæumsfond*

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CYTOLOGICAL AND HISTOLOGICAL EVENTS FOLLOWING TREATMENT WITH ANTI-THYMOCYTIC GLOBULIN IN MICE

Studies on the increased decay in lymphatic tissue

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The present experiment was undertaken in order to obtain additional information on the increased cell decay in lymphatic tissues after ATS treatment After injection of rabbit anti-mouse globulin G (ALG) in Balb/c mice an increased cell decay was found in spleens and mesenteric nodes by the use of the Nigrosin Dye Exclusion Test on unfixed cell suspensions After one injection of ALG the decay increased to the same high level as seen after ATS-injection no further increase being found after 7 days of injections One week after treatment was stopped, increased decay was still evident In suspensions from the thymus the decay remained on normal level Mice injected with normal rabbit globulin G showed no increased decay in the thymolymphatic system The most prominent histological findings were depletion of the thymus dependent areas in lymphatic tissue and increased numbers of blast-cells in the blood and efferent lymphatics after ALG injections The possible ways in which treatment with anti lymphocytic antibodies may lead to increased decay in lymphatic tissue are discussed in connection with the other findings and it is concluded that the most likely explanation is a shift in population from long lived thymus derived cells to short lived cells in lymphatic tissue and it is emphasized that the Nigrosin Dye Exclusion Test is a useful test in studies on lymphoid cell kinetics

e in vivo immunosuppressive effects of an-lymphocytic serum (ALS) have been widely ied and are well documented, but the chanism by which it achieves these effects unsolved

Most observations lend support to the hypo-is that the longlived recirculating, thy-s derived lymphocyte is the primary target cell, and the varying histological findings – hypercellularity or depletion of "thymic dependent" areas – can be explained by blastic transformation of small lymphocytes or selective destruction of thymus derived lymphocytes induced by ALS (3, 9, 23, 27, 31, 32 34) Perhaps an additional effect of ALS on the reticuloendothelial system exists (4,15,19,25 26) but the importance of this is less clear

In a previous study (30), increased numbers of nonviable lymphocytes were found in mice lymph nodes after a single injection of antithymocytic serum (ATS) It was cor

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cluded that the most likely explanation of this was *a shift in population from longlived to* shortlived lymphocytes

The present experiment was undertaken in order to obtain additional information about this effect of ATS Immune globulin G (ALG) was used instead of total serum for injections to avoid unspecific serum effects In addition, the effects of prolonged treatment with ALG were investigated by use of the Dye Exclusion Test and histological exam inations *of the thymolymphatic system*

MATERIALS AND METHODS

The ATS was raised against Balb/c thymocytes in white Danish country rabbits The method used for raising the antisera was that described by *Levey* and *Medawar* (23)

The IGG from ATS (ALG) and normal serum (NRG) was prepared as follows The ATS was salted out with 250 g ammonium sulphate per litre of ATS After dialysis DEAE A50 Sephadex chromatography was performed at pH 5 0 and ion strength = 0 05 The eluate was concentrated by renewed salting out Finally the product was dialysed against 0 1M Sodium chloride and 15 mM Sodium azide NRG from normal rabbit serum was prepared in the same manner It was stored at 4°C until use

The leuco-agglutination test was performed as described by *Abaza* & *Woodruff* (1) and percent age of agglutination was calculated as 100 x (total number of cells free cells)/total cells Titre was expressed as the reciprocal of the highest di lution giving more than 20 per *cent agglutination* and was found to be 1/256 All control values were less than 10 per cent

The immunosuppressive effect of the ALG was *tested by grafting Balb/c mice with* C_3H *skin* Two subcutaneous injections of 5 mg ALG given 2 and 5 days after transplantation were capable of prolonging the median graft survival time from 10 to 22 days

Eighty 4 5 week old Balb/c mice (received spe cific pathogen free from Bolholtgård Laven) were examined Equal numbers of males and females were used During the experiment the animals were kept under standard conditions

Ten animals were not injected and were exam ined as controls during the entire experimental period

The remaining animals were divided into two groups The first group received ALG injections in the subcutis of the infrascapular region and the other received NRG injections in the same area

Ten mice from each group were injected with *globulin doses equivalent to 0 5 ml of total serum* at day 0 and were examined 24 hours later (day 1)

Twenty five mice from each group were injected as mentioned above, but on day 1 and the next 5 days they were injected in *the subcutis of the* infrascapular region with globulin doses equivalent to 1/4 ml of total serum.

Ten animals from each group were examined on both day 8 and day 14 On day 21, 5 mice from each of the two groups were sacrificed and organs removed for histological purposes

All animals except the ones in the 21 day group were examined as follows The animal was weighed and blood was taken from the intra orbital plexus of veins WBC and differential white cell counts were done later The animal was then killed by cervical dislocation in ether anaesthesia

The thymus spleen and mesenteric lymph node were removed and placed in chilled Hanks solution The organs were weighed and parts of each organ were placed in Lillies fixative or in glutaraldehyde and osmic acid Later they were embedded in pa raffin and epon and cut in 4 and 1 micron sections

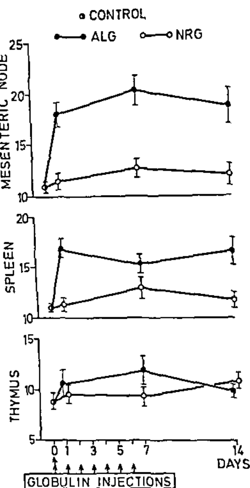
The paraffin sections were stained with both methylgreen pyronine (controlled with ribonuc lease) *and haematoxylin and eosin The epon* sec tions were stained with toluidine blue Peyer's patches and inguinal nodes were removed from a few mice in each group placed in the same fixativ es as the above mentioned organs and examined in the same manner Slides of bone marrow sus pensions were made from animals in each group and were stained with Romanowsky stain

Single cell suspensions in Hanks solution were prepared from the thymus the mesenteric node and the spleen immediately after the organs had been weighed and tissue parts removed for microscopy The spleen was first flushed with Hanks solution to avoid excessive contamination with red blood cells The suspensions were made by mincing the tissue with scissors on siliconed glass placed on ice The Nigrosin dye exclusion tests was then per formed on *the cell suspension The technique of* the Nigrosin dye exclusion test is described in a previous paper (30)

RESULTS

Dye Exclusion Test

Fig 1 shows the variation in stained (non viable) cells in free cell suspensions from thymuses, spleens and mesenteric nodes in non injected NRG treated and ALG-treated mice No significant variations occur in the percentage of stained cells in thymus suspensions whether the animals were ALG injected



1 Changes in the number of non viable cells single-cell suspensions from mesenteric nodes, , and thymuses Control = non-injected mice, G = mice injected with anti lymphoid globulin, = mice injected with normal rabbit globulin , indicate injection of a globulin dose equivalent to 0 25 ml of total serum. Bars represent standard error of the mean

NRG injected, at the days of examinations 7 and 14 days after the start of the 7 days irse of treatment In suspensions from ens and mesenteric nodes in ALG-treated a sudden increase in non-viable cells is n the first day following the start of in-ions The percentage stays at the increased el on day 7 and 14 In the suspensions from treated animals, the percentage remains l levels There seems to be a slight in-ase on day 7, but this is not significant

Weight Measurements

As seen in Fig. 2, relative spleen weight decreased ($p < 0.05$) 24h after subcutaneous administration of ALG It increases to normal values by day 7 in spite of continued ALG treatment, and it is still normal on day 14 – one week after treatment has stopped The relative mesenteric weight from mice treated with ALG seems to be less than that from NRG treated mice on day 1 and 7 but the differences are not significant On day 7 the relative weight of thymus in the ALG treated group is depressed ($p < 0.01$) but returns to a normal value on day 14 (Fig 2). No sign-

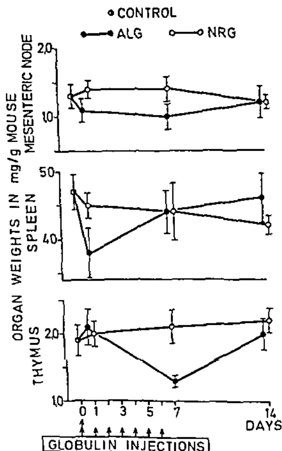


Fig 2 Relative organ weights from mice treated with globulin Control = non injected mice, ALG = mice injected with anti lymphoid globulin, NRG = mice injected with normal rabbit globulin. Arrows indicate injection of a globulin dose equivalent to 0 25 ml of total serum Bars represent 2x standard error of the mean

ificant variations occur in the relative organ weights from NRG treated animals

Blood lymphocytes

The first day after injection of ALG the mean of the blood lymphocytes was 600 per mm^3 but increased to 2100 per mm^3 by day 7 in spite of the continous treatment By day 14 the total lymphocyte count had returned to normal On day 1 only a few blastlike lymphocytes were found but by day 7 the smears showed significant increase in blast forms – lymphoblasts as well as granuloblasts These forms were still prominent at day 14 but few remained by day 28 These results were confirmed by counts of blastcells in the efferent lymphatics of mesenteric nodes in methylgreen pyronine stained sections There were 5 to 10 lymphoblasts per 100 lymphocytes in sections from untreated mice One 7 and 14 days after the start of ALG injections the numbers per 100 lymphocytes were 10–20 20–50 and 20–40 respectively

The total lymphocyte counts from NRG treated mice stayed at normal levels and except for minor increases in the numbers of blastlike lymphocytes on day 7 nothing of interest was seen

Histology

The first day after the start of treatment with ALG no changes were seen in the thymus architecture and no depletion was seen in the cortex or medulla The mesenteric and the inguinal nodes showed depletion of lymphocytes in the peripheral regions of cortical modules and paracortical layer The degree of depletion was less than that seen after total serum treatment The peripheral part of the white pulp of the spleen showed decreased numbers of lymphocytes The NRG treated animals compared favorably to controls

On day 7 the day after the 7 day course of ALG treatment the depletion seen in lymph nodes and spleen (Fig 3 and 4) was more prominent that on day 1 and blastforms of lymphocytes were seen in the depleted para cortical layer Germinal centres were always seen and the number of mitoses did not differ from the number in NRG treated mice In the latter blastcells were observed but with the exception of this and the prominent germinal centres no evident histological changes were observed The thymuses from ALG treated mice showed varying depletion of both cortex and medulla In the latter increased numbers of pycnoses were observed

Day 14 sections from the lymphatic tissue of ALG treated mice showed no signs of normalization The depletion was still evident and in addition necroses and pycnoses were seen in the mesenteric nodes and Peyer s patches Depletion here as in the 7 day group was especially evident around the follicular artery in the spleen Blastforms of lymphocytes were still prominent in the depleted areas

The morphology of the thymuses in this group appeared normal The 14 day group of NRG injected mice did not differ essentially from the 7 day group

Day 21 showed a repopulation of the depleted areas in the ALG treated animals but necroses were still apparent In the thymus numerous Hassall s bodies – an expression of cell degeneration (5) were observed compared to the very few seen in untreated Balb/c mice

No constant feature was seen in the red pulp of the spleen In some ALG treated mice in the 7 and 14 day group high numbers of lymphoblasts were seen In the NRG treated groups lymphocytes in the red pulp were nearly always mature

In smears from bone marrow increased numbers of immature polymorphonuclear cells were seen in ALG treated animals The number of lymphocytes did not seem to differ from NRG treated or control animals

DISCUSSION

The Nigrosin Dye Exclusion Test has been established as a valuable *in vitro* test for determining dead or dying cells by their capacity for staining by Nigrosin In the present and earlier papers from this laboratory *(6 7 30)*

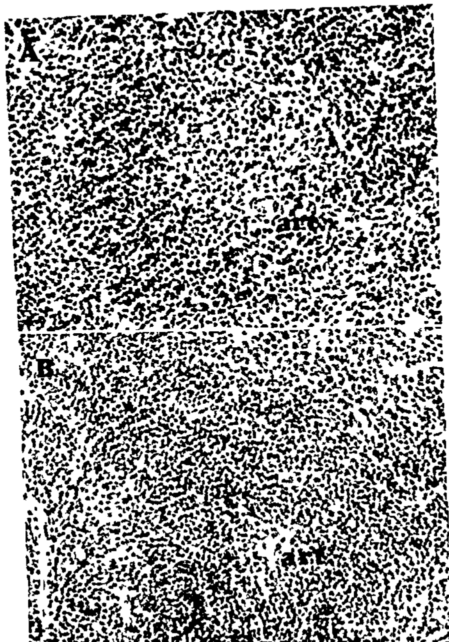


Fig 3 3A Splenic white pulp with follicular artery (art) from a mouse killed one day after 7 days of ALG treatment 3B Splenic white pulp with follicular artery (art) from a mouse killed one day after 7 days of NRG treatment Note the periarteriolar depletion in 3A compared to 3B Haematoxylin and eosin (400 ×)

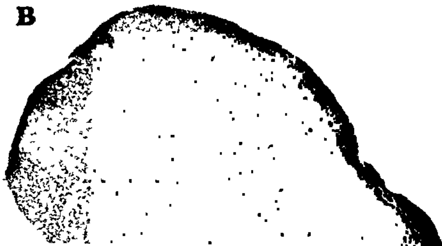
T is used in the evaluation of lymphoid l kinetics

must be taken so that the cells are not naged during preparation of the suspensions and the staining time, i e the time interval from uptake of Nigrosin until appearance of the cells, must not vary appreciably in the different suspensions

A



B



C



Fig 4 4A and 4B Mesenteric- and inguinal lymph nodes from a mouse killed 1 day after 7 days of ALG-treatment showing paracortical depletion. 4C Mesenteric lymph node from a mouse killed 1 day after 7 days of NRG-treatment showing normal architecture Haematoxylin and eosin (× 40)

ions are met, an increased number of ned cells will indicate increased cellular , which may be due to either increased or a shift in population from long-ed to short lived cells When a suspension of hocytes is incubated with ALG and com t an increased number of decaying cells seen *Röpke* (30) demonstrated that cell struction was not increased in thymus sus , from mice treated with ATS and, as here, no increased decay is seen in suspensions after treatment with ALG finding may be explained by the fact ALG penetrates thymus to a very small gree (12, 21) and that very few cells which ay have been exposed to ALG elsewhere the thymus (28)

After ATS injection an increased decay of mphocytes is seen in suspensions from me nteric and mixed axillary/inguinal nodes) The same is true in suspensions from and mesenteric nodes after treatment th ALG, indicating that increased decay nnot be a nonspecific serum effect The ay in lymphatic tissue is not increased by jections of NRG which confirms that the is due to antibodies

The increased decay cannot be explained a direct cytotoxic effect of ALG since the etration of ALG into lymphatic tissue has shown to be negligible (10, 21) It has iously been shown that the number of *ned cells* is not increased during the first after injection of ATS (30) In the sent study an increased decay is still found days after treatment with ALG and since *LG* is known to be eliminated in 3 days (21) he evidence seems to be against a direct cyto-oxic effect of ALG in lymphatic tissue as a of the increased decay

A decline in the blood lymphocytes is in uced by injection of ALG and is attributed a selective killing of circulating thymus-ved, lymphoid cells, (3, 9, 27, 31, 34) hese cells are principally cleared from the lood in the liver *(26, 27, 32), thus an up-* of damaged blood lymphocytes cannot be he explanation of the increased decay after LG injection

Injection of steroids results in an increased decay in the thymolymphatic system (7) In creased *levels of corticosterone are found in* the blood a few hours after injection of ATS (18), and this may also be a contributing factor in the increased decay after ALG injection However, thymus is especially sensitive to cortisol (7, 13) and as no increased decay is found in suspensions from thymus, it does not seem probable that the increased content of corticosterone in the blood is the cause of the increased decay observed

Since the increased number of stained cells in suspensions from lymphatic tissue is not due to a direct toxic effect of ALG, it is most obvious to consider the increased decay as an indication of a shift from long lived to short lived cells Several findings support this hypothesis The bone marrow seems unaffected by ALG injection and delivers increased numbers of short lived cells into the blood (9) where they are not damaged by ALG An increased proliferation of short lived cells in lymph nodes has been found after ALG injection (11) and most of these cells do not recirculate but remain in the lymphatic tissue The increased decay may thus be attributed to a dominance of short lived cells, since the long lived, thymus-derived recirculating cells have been eliminated (31)

The changes in weight and the histological findings during and after ALG treatment are consistent with this assumption The decrease in weight of the spleen on the first day of treatment may be due to an inhibited supply of recirculating cells while the output to the blood continues Some *experimental findings* indicate a decreased uptake of thymus derived lymphocytes in lymphatic tissue after injection of ATS (30), and lymphocytes damaged by ALG, which make up the greater part of the blood *lymphocytes (8, 14, 20)*, are princip ally found in the liver It has not been deter mined whether *the output* of cells continues at the normal rate, but the thinning out of the *white pulp* of the *spleen* seems to support this hypothesis The proliferation of short lived cells *may explain* the normalization of the weight in spite of continued treatment (11)

Marshall & Knight (25) have found the spleen weight to be increased after injection of ALS, whereas other authors do not find any weight changes (19) These findings show that differences between different antisera or between total serum and ALG may exist Irrelevant antibodies – especially hemolysins – must be considered In this respect it must be noted that different antisera give rise to varying output of cells from the thoracic duct (27, 30, 34) If the output of cells from the spleen into the blood varies with different antisera, this might explain the different spleen weights.

The mesenteric node – unlike the spleen does not exibit a significant weight decrease on the first day This may be explained by the fact that lymphocytes remain here for a longer time *Ford & Gowans* states the average transit time to be 5–6 hr in the spleen but 15–20 hr in lymph nodes (16) It must be noted that Tyler et al (34), in spite of weight constancy, found a decrease in DNA content in mesenteric nodes after ATS injection

Denman et al (12) found both thinning out of the cortex and medulla of the thymus as well as a decrease of weight of the thymus after 7 days of treatment These effects of treatment are also found in the present investigation and can be explained if thymocytes are released into the blood in greater numbers than normally since the production is not impaired

The fact that the weight of the thymus increases to normal level one week later proves that the balance is quickly restored when ALG is eliminated and the blood lymphocytes reach normal levels

The histological findings vary greatly after treatment with antiserum The failure of various authors to obtain uniform results might be correlated to the presence of irrelevant antibodies as suggested by *Levey & Medawar* (24) The varying time intervals between injection and investigation may also be of importance The histological findings after injection of ALG as reported here are comparable to the findings by other authors (2 18, 24, 30, 33, 34) The most notable finding is the thinning out of the paracortical area in the lymph nodes and the periarteriolar area of the white pulp of the spleen – the so called thymus dependent areas – which are also thinned out after neonatal thymectomy (29) In these areas and in the blood – especially on day 7 – an increased amount of immature cells is found This finding has been described by different authors (4, 17, 24, 34) Together with the transformation of lymphocytes into blasts *in vitro* in the absence of complement it forms the basis for the so-called sterile activation theory (23) This theory may partly explain the mode of action of ALG

It is also considered of interest that Peyers patches – thought to be an equivalent of the bursa are thinned out just as the lymph nodes and the spleen

As shown here necroses are seen in the thymolymphatic system after treatment for a longer period of time This phenomenon especially involves the reticular cells – but does not occur consistently The rationale of this long time effect of antisera which has also been described by others (18) has not been clarified The reticular cells make up about 2 per cent of the cells in the suspensions and an increased decay of these cells cannot be measured by means of the method here employed

The conclusion of this and the preceeding paper (30) is that the Nigrosin Dye Exclusion Test is a useful test in lymphoid cell kinetics The increased decay in lymphatic tissue measured by the use of the test indicates that treatment with ALG induces an elimination of long lived cells It is not clear whether the increased decay in the lymphatic tissue after treatment with ALG can be correlated to the immunosuppressive effect of ALG

I am most thankful to dr *Niels Axelsen* The Protein Laboratory University of Copenhagen for preparing the IgG

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AN EXPLANATION OF THE BIOLOGICAL ACTION OF TOXOTOXIN BASED ON SOME *IN VITRO* EXPERIMENTS

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The present investigation has failed to show any correlation between the lethal activity of Toxotoxin and the viscosity of its solutions *In vitro* experiments in the presence of mouse plasma revealed a dual character of Toxotoxin, this being partly thromboplastin and partly coprecipitant to fibrin *In vivo*, the thromboplastic activity, though in itself checked by fibrinolysis, was thought to initiate the coprecipitation of Toxotoxin, thus causing the formation of a massive clot which fibrinolysis would be unable to resolve

nman & *Klatchko* reported their dis-ry of Toxotoxin in 1950 and referred to t was thought to be a toxin of *Toxo-gondii* (8) Toxotoxin was demon-ed by intravenous injection into mice of free peritoneal exudate obtained from with severe experimental toxoplasmosis mice died usually within the first min-of injection Sublethal doses produced sions, hyperpnoea, and sometimes quarter paralysis After recovery the appeared to be normal (10)

he lethal effect could be enhanced by ing the exudate at 56° C (9) As the osity of the exudate was increased at the time some workers were led to believe the lethal activity was due to the visco-11) or to a combination of viscosity and tension (4)

on demonstrated the presence of Toxo in the exudate obtained from cotton (1) The exudate was shown to have no properties in common with a true toxin The lethal activity was correlated with a molecular entity of protein hyaluronate nature causing circulatory disturbances This was based partly an autopsy results corroborated by other workers (4), showing a massive clot in the right ventricle of the heart

Immune serum did not offer any protection against the lethal activity of Toxotoxin (1) which immunochemically was shown to be a product of the host rather than of the parasite (5)

In order to arrive at a plausible explanation for the mode of action of Toxotoxin, the present investigation of a possible correlation between the lethal activity and physical/chemical properties of Toxotoxin was undertaken

MATERIALS AND METHODS

Toxotoxin solutions The preparation of crude exudate, heat potentiated exudate, "ice filtration" residue, and ammonium sulphate precipitate was

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according to procedures previously described (6). All Toxotoxin solutions used in this study were centrifuged at 50 000 *xg* for 10–30 minutes before use.

Plasma. Mouse blood containing 10 per cent by volume of a 10 per cent sodium citrate solution was centrifuged at 1,000 *xg* for 30 minutes. The platelet rich supernatant was used for the determination of the thromboplastic activity. Platelets were removed by recentrifugation at 27,000 *xg* for 10 minutes for the quantitative precipitation determinations.

Buffers. The phosphate buffer was M/20 with respect to sodium phosphate and M/10 with respect to sodium chloride. The pH was 7.50. The buffered saline was a 0.9 per cent sodium chloride solution which was *M/2,000* with respect to sodium phosphate. The pH was 7.5.

Viscosity. The viscosity was measured on dust free solutions obtained by centrifugation at 50 000 *xg* for 30 minutes. The relative viscosity was calculated according to the formula $n_r = \rho t/t_0$ where t was the time of flow in an Ostwald viscometer of the solution to be tested, t_0 was the corresponding time of flow for distilled water, and ρ was the specific gravity of the solution to be tested. The measurements were made at room temperature.

Coagulation. The solution to be tested was mixed with an equal volume of citrated mouse plasma at 37° C. Half a volume of a 1.7 per cent calcium chloride solution was added and a record made of the time required for a clot to form.

For the quantitative determination of the precipitation involved in the coagulation experiments the tube was agitated until the precipitation was completed. The tube was then centrifuged at 27,000 *xg* for 30 minutes. The sediment was washed with distilled water, dried at 110° C and weighed.

Solution of the 'ice filtration residue' was dialysed against buffered saline before use in order to remove all but a trace of phosphate ions. The buffered saline was also used as a control in these coagulation experiments.

Biological assay. The Toxotoxin solutions were injected intravenously into the tails of three week old white mice of the institute's own strain weighing approximately 18 g. The minimum lethal dose was the minimum dose that killed the mice within five minutes of injection. Doses were graduated by 0.05 ml from 0.05 ml to 0.5 ml.

RESULTS

When the crude exudate was heated at 56°C in order to potentiate its lethal activity, a coagulation of proteineous matter took place making the exudate very viscous. After centrifugation, however, the viscosity was the same as that of the crude exudate (Table 1). Viscosity measurements were also made on other Toxotoxin solutions, and the results obtained were compared with the lethality of the solutions in question (Table 2).

TABLE 1. *Effect of Heat Potentiation on the Minimum Lethal Dose of Peritoneal Exudate*

Exudate	No.	Minimum lethal dose in ml	Relative viscosity
Crude exudate	1	0.4	1.86
	2	0.4	1.98
	3	0.35	1.84
Heat potentiated exudate	1	0.05	1.83
	2	0.05	1.90
	3	0.05	1.79

The biological assay gave very much the same reaction as that described by *Weinman & Klatchko* (8). Though the sublethal doses of crude exudate affected the respiration and movements of the mice, it was found that the sublethal doses of heat potentiated exudate rarely had any effect at all.

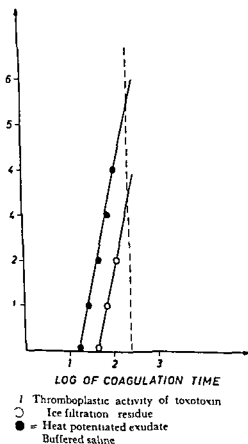
The exudate was readily miscible with mouse plasma. No precipitate was formed and the viscosity remained approximately the same after mixing. Coagulation took place after the addition of calcium ions, and at a higher rate in the presence of exudate than in its absence. This is illustrated in Fig. 1,

TABLE 2. *Comparison of Lethality and Viscosity of Toxotoxin Solutions*

	Minimum lethal dose in ml	Relative viscosity
Crude exudate	0.4	1.86
Heat potentiated exudate	0.05	1.83
Ammonium sulphate precipitate	>0.5	1.35
Heat potentiated ammonium sulphate precipitate	0.2	1.32
Ice filtration residue	0.1	1.19

TABLF 3 *Dry Weight Precipitate per ml Toxotoxin Solution*

le	No	Sample + plasma + calcium (mg)	Saline + plasma + calcium (mg)	Sample + saline + calcium (mg)	Coprecipitate (mg)	Sample concentration (mg/ml)	Minimum lethal dose (ml)
	1	1 87	1 23	0	0 64	0 55	0 1
ion residue	2	1 44	1 41	0	1 03	0 86	0 1
	3	1 47	1 03	0	0 44	0 51	0 15
	1	2 53	1 43	0 38	0 72		0 4
te	2	2 22	1 27	0 36	0 69		0 4
	1	5 99	1 43	0 36	4 20		0 05
tiated exudate	2	5 42	1 27	0 43	3 7?		0 05



I Thromboplastic activity of toxotoxin
○ Ice filtration residue
● = Heat potentiated exudate
Buffered saline

ich shows a straight line relationship be en the logarithms of coagulation time and procal dilution (3) Crude exudate had itly higher thromboplastic activity than heat potentiated exudate

\gitation of the plasma exudate calcium mixture gave a fibrinous precipitate, the bulk of which was larger than could be acounted for by fibrin formation alone A similar result was obtained when a solution of 'ice filtration residue was used instead of exu date (Table 3) The solutions left after precipitation had no lethal effect on mice

DISCUSSION

The results given in Tables 1 and 2 demonstrate the lack of correlation between the lethal activity of Toxotoxin and the viscosity of its solutions This is contrary to the results obtained by *Nozik & O Connor*, who claimed that the lethal activity could be correlated with the viscosity and surface tension characteristics of the exudate (4) They based their conclusion on viscosity measurements of a heat potentiated exudate which had not been centrifuged It is, therefore, apparently possible that the increase in viscosity found by them could be accounted for by the presence of coagulated proteineous matter The sucrose solution with which they compared the properties of the exudate, would more likely be lethal due to its hypertonicity rather than to its viscosity The difference in surface tension between mouse serum and heat potentiated exudate by an estimation from the rise in parallel capillaries appeared to be insignificant in this connection

On the basis of previous (6) and present results, Toxotoxin can be described as a large, fibrinous molecule or particle formed

by the aggregation of smaller molecules, some of which contain thromboplastic activity (Fig 1) The thromboplastic activity suggests the presence of lipoprotein (2) which, in fact, was found to be an integral part of Toxotoxin (7) The thromboplastic activity did not increase in the process of heat potentiation, thus excluding its possible correlation with the lethal activity of Toxotoxin.

Quantitative analysis of the dry matter involved in the coagulation of mouse plasma combined with Toxotoxin showed an excess beyond the amount accounted for by fibrin This excess was several times larger with heat potentiated exudate than with crude exudate (Table 3), thus indicating a correlation between the degree of coprecipitation and the lethal activity of the exudate The excess obtained with the solution of "ice filtration" residue could be accounted for by the amount of residue added (Table 3) In addition, the fact that the supernatant solution from these coagulation experiments had no lethal activity suggests that the material that killed the mice was the same as that which coprecipitated with fibrin

From a comparison of these results with the results of the biological assay, it appeared likely that the sublethal doses of crude exudate that caused transient hyperpnoea and convulsions corresponded to the thromboplastic activity of Toxotoxin The mice appeared to be quite normal after recovery, which probably could be due to the formation of intravascular clots small enough to be resolved by fibrinolysis

Sublethal doses of the heat potentiated exudate did not have a sufficiently high concentration of thromboplastin to give a noticeable reaction in the mice The minimum lethal dose would correspond to a thromboplastic activity, not necessarily noticeable in itself, but large enough to initiate a coagulation process which, when grossly substantiated by the coprecipitation of Toxotoxin, would form a clot too large to be resolved by fibrinolysis before it killed the mouse

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In part aided by grants to the Department of Toxoplasmosis and Viral Diseases from *King Christian X Foundation* and the *US Public Health Service Grant Foundation* (Ai 1741), *Bethesda Md*

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Acta path microbiol scand Section B 79, 37–50, 1971

ELECTRON MICROSCOPY OF ENDOFLAGELLA AND MICROTUBULES IN *TREPONEMA* REITER

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Treponema Reiter was studied by negative staining and ultrathin sectioning techniques after treatment with the detergents Teepol and sodium deoxycholate, and with the proteolytic enzyme AL-1 Special attention was paid to the insertion ends of the endo flagella and the structures revealed have been compared with those published by other workers on the attachment ends of bacterial flagella of various species All the treatments freed the flagella from the treponeme though the basal discs were often obscured by mem brane fragments adhering to them The endoflagella were found to consist of the following structural parts 1) a sheathed shaft, 2) a hook that differs from the shaft both in width [illegible]

[illegible]

terminate close to the flagellar basal discs at each end of the cells The two bundles of microtubules overlap in the middle of the organism In sectioned cells these microtubules were seen in the cytoplasm close to the inner layer of the cytoplasmic membrane and always directly under the place where the endoflagella were found in the interspace be tween this membrane and the cell wall Each individual microtubule showed a diameter of about 75 Å both in negatively stained and in sectioned material

[illegible]cent report on the morphology of *Tre*[illegible]*ma pallidum* Nichols (11) indicated that [illegible] was structural evidence that the intra[illegible]lar fibrils of this treponeme could [illegible]idoflagella Further observations were [illegible] however, for a comparison of the [illegible]ucture of the insertion ends of these [illegible]agella with the structures resolved in [illegible] of bacterial flagella (1, 2, 5, 8) [illegible]ldition it was considered worthwhile to compare the substructure of the *insertion* region with that of the corresponding part of the axial fibres of some species of *Leptospira* (4, 17)

Treponema pallidum Nichols can be propagated only by intratesticular passage in the rabbit, and it proved to be difficult to obtain organisms in sufficient amounts to yield a reasonable number of free flagella for study Consequently we decided to use *Treponema* Reiter for some preliminary studies This organism can be grown in artificial media, it has from five to seven endoflagella inserted at each end (10, 18, 22), whereas *Treponema pallidum* Nichols has only three (11)

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The results of the studies on the endo flagella and their insertion into the cyto plasmic body of *Treponema* Reiter are pre sented in this paper These studies have pro vided a good basis for comparing these organelles and their attachment with those of the bacteria mentioned above Evidence for the presence of two bundles of intra cytoplasmic microtubules in the Reiter tre-poneme is also reported

MATERIALS AND METHODS

Treponema Reiter was obtained from the Trepone matoses Department Statens Seruminstitut, Co penhagen The strain was originally provided by Dr de Bruijn, Rijksinstituut Utrecht The Nether lands Cells were grown at 36° C in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 on a thio glycollate medium of the following composition per litre 15 g of a pancreatic digest of casein (Shef field Chemical Norwich New York), 0 75 g L-cystine 5 5 g glucose 5 g yeast extract and 0 5 g sodium thioglycollate with 10 per cent human serum added The cells were harvested after 5 days by adding one volume of SMC (a sucrose solution containing magnesium and calcium ions[1]) to one volume of the cell suspension, and subse quent centrifugation for 10 15 minutes at 2000 × g (7–8000 rpm in a Christ junior 15000 table model centrifuge) The pellets thus obtained were generally resuspended in a few drops of SMC and the organisms were immediately treated as stated below

Preparations for Negative Staining

The treponemes were treated on specimen grids for electron microscopy with 0 2 per cent Teepol or 1 per cent sodium deoxycholate both in re distilled water or with a solution of AL-1 en zyme containing 100 µg/ml of the enzyme and 5×10^{-4} M of EDTA in 2.5×10^{-3} M TRIS buffer pH 9 0 (5)

The procedure used for treatment and negative staining was a multiple drop technique Drops of 1) organisms suspended in SMC 2) detergents or enzyme, and 3) the negative stains used were placed on a piece of dental wax A carbon rein forced Formvar coated grid was placed on top of the drop containing organisms in SMC with the Formvar film facing downwards After about one minute the grid was taken up and surplus fluid was removed with a piece of filter paper, exten sive drying was avoided The grid was immediately placed on the active agent for the desired period of time Again surplus fluid was removed, and the grid was floated on a drop of the stain After 1–2 minutes the grid was removed, dried carefully with a piece of filter paper and was now ready for elec tron microscopy The time used for treatment with the active agents varied from 30 seconds to 4 minutes

The stains used were
1) 1 per cent (w/v) ammonium molybdate ad justed to pH 7 with NH_4OH
2) 1 per cent (w/v) lithium tungstate adjusted to pH 7 with LiOH
3) 1 per cent (w/v) uranyl formate dissolved in 1 per cent ammonium acetate and adjusted to pH 6 with NH_4OH

Some cells were treated with sodium deoxycholate by suspending a pellet of organisms in redistilled water and adding 1 per cent sodium deoxycholate to a final concentration of 0 5 per cent This suspension was left at room temperature for 30 minutes before centrifuging for 10 minutes at 3000 rpm (Christ centrifuge) to remove coarser debris The supernatant was centrifuged for 30 minutes at 114 000 × g (40 000 rpm in a preparative ultra centrifuge Spinco Model L) The pellet was washed twice in redistilled water by repeated high speed centrifugation Negatively stained prepara tions were made at all steps

Treatment of cells in suspension was also carried out by resuspending the pellet of organisms in a solution containing 100 µg/ml AL 1 enzyme and 5×10^{-4} M EDTA in 2.5×10^{-3} M TRIS buffer pH 9 0 The suspension was left at room temperature for 30 minutes By then the optical density read at 550 nm showed a drop in density of 47 per cent Prolonged enzyme treatment did not decrease the optical density value The sus pension was centrifuged for 30 minutes at 96 000 × g and negatively stained preparations were made from pellets thus obtained

Preparations for Sectioning

10 ml of SMC + 1 ml 3 per cent glutaraldehyd in redistilled water were added to 10 ml of 5 day old cells in their culture medium and pre fixed for 30 minutes inclusive 10 minutes centrifugation at 7–8000 rpm (2000 × g) The pellet was enrobed in warm melted agar at 45° C (1 5 per cent Noble Agar Difco in SMC) Agar blocks of about 1 mm³

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[1] The SMC solution consisted of 0 03 per cent sucrose, 0 01 M $MgCl_2$ and 0 01 M $CaCl_2$ in redistilled water The pH was not adjusted but was generally about pH 5

[2] The purified bacteriolytic enzyme of myxo-bacterium AL 1 was kindly provided by Prof R S Wolfe, Department of Microbiology Uni versity of Illinois Urbana Illinois USA

and fixed overnight at room temperature cent OsO_4 in SMC to which was added r cent YAP medium (yeast extract—sodium —peptone medium 0 3 0 05 and 0 3 per respectively of the Difco products) After a wash in SMC the blocks were treated for 1 at room temperature with 2 per cent uranyl e in SMC (20) then dehydrated in alcohol lene oxide (16) and finally embedded in pal W (21)

some specimens the osmium fixation was Instead the agar blocks were postfixed ½ hours at room temperature in 3 per cent yde in SMC After a brief wash in the blocks were treated for 1 hour at room ature with 1 per cent uranyl acetate in Prior to embedding after propylene oxide t as mentioned above these blocks were drated in a series of increasing alcohol con ations up to 100 per cent saturated with l acetate in all alcohol concentrations used

oning

in sections were obtained on the LKB I and III microtomes Normally the were poststained by floating grids section vn on magnesium uranyl acetate (6) and ards on lead citrate (19) Sections from treated with uranyl acetate during dehydra were however poststained only with lead e diluted 1 10 with redistilled water

ctron microscopy was carried out on a Philips 00 or a Philips EM 300 electron microscope imary magnifications of 9000 × 16 000 × 000 × Negatives were obtained on Kodak Grain Release Positive Film Type 5302, and photographically enlarged as desired For approximately 1200 recordings were

RESULTS

rvations on Negatively Stained Material

ter 30 seconds of treatment with 0 2 per Teepol in redistilled water the helical ne of *Treponema* Reiter is still evident cytoplasmic body of the organism has a teristic spotted appearance (Fig 1) of the endoflagella are no longer in d into the cytoplasm of the organism flagella are often seen to wind around organism although the hooks and basal generally are detached from the inser region (Fig 1) On such liberated flag the end region is seen to consist of a shaft proper, a hook and a basal disc (Figs 2, 3) The shaft of the flagellum has a diameter of 180 Å An abrupt change in the fine structure is obvious where the hook starts (Fig 3) The substructure of the hook is honeycombed and thus different from the pattern of sub units in the shaft (Fig 3) The diameter of the hook is 140 Å A narrow collar connects the tip of the hook to the basal disc This narrow collar has a diameter of 95 Å and is 120 Å long The substructure of this part is different from that of the hook proper (Fig 2) The structure and size of the basal organelle is difficult to resolve in Teepol-treated organisms because the organelle was frequently found to be more or less obscured by attached remnants of membranes

Flagella were also isolated by differential centrifugation after treatment of cells in suspension with 0 5 per cent sodium deoxycholate The substructure of the flagella was as mentioned above In this study we consider the end of the flagellum with the basal disc as the proximal part whereas structures observed along the length of the flagellum are considered distally positioned The distal part of the hook shows a honeycombed substructure while the proximal end appears smooth and somewhat thinner (Figs 4 5) The basal knob is still quite difficult to resolve, but seems to consist of a single disc shaped plate (Figs 4 5) The diameter of this is approximately 270 Å

The shaft on flagella isolated from this type of preparation can be seen to be covered by a sheath Sometimes the sheath can be removed by washing the isolated flagella repeatedly with redistilled water The naked flagellum has a diameter of 120 Å Such a naked flagellum is seen in Fig 6 together with an ordinary sheathed flagellum The sheath was found to have a helical substructure when subjected to optical diffraction[a]

[a] The optical diffraction was carried out by Prof *R Horne* the Johns Innes Institute the University of East Anglia Norwich U K, and Prof *R G E Murray* Department of Bacteriology University of Western Ontario, London, Ontario Canada during the visit of the latter to Cambridge in April 1969

Treatment of cells on the grid with 1 per cent sodium deoxycholate reveals bundles of thin filaments in the cell interior (Figs 7, 8 9) Closer inspection showed clearly, however, that these filaments are hollow structures (Fig 18), and they are thus considered to be microtubules for the purpose of this article A bundle of microtubules extends from each end of the organism These two bundles overlap in the middle of the cytoplasmic body, and each bundle seems to wind around the organism (Figs 7, 8) The diameter of each individual microtubule is about 75 Å

The helical outline of the cell as seen after sodium deoxycholate treatment is distorted and quite irregular (Figs 7, 8, 9) Many flagella are still inserted into the cytoplasmic remains of the organism after 2–4 minutes of treatment (Fig 9)

Flagella that are liberated after Teepol or sodium deoxycholate treatment present a wavelength and an amplitude nearly identical to those of flagella in untreated and undamaged organisms whereas the flagella appear to be straighter after treatment with AL-1 enzyme In addition, flagella show a tendency to break after enzyme treatment

Treatment with AL 1 enzyme for 30 seconds completely destroys the helical form of the treponemes Only flagella bundles of microtubules, and some membranous debris are left on the grids (Fig 10)

The flagella are often found attached to some portions of membrane As many as 8 flagella can be observed attached to the same membrane fragment (Fig 11) The hook with the honeycombed substructure and the smooth and narrow collar is clearly depicted (Fig 12) The basal disc appears to be single and is generally connected to membrane fragments (Figs 12 13) In the latter, ring shaped structures seem to surround the basal discs (Figs 14 15)

Frequently the proximal ends of the microtubules are found close to the insertion point of the flagella (Fig 16) 6–8 microtubules are present at each end of an organism (Figs 14 17) As many as 16 individual tubules can be observed in the central part of the cell (Fig 18) The microtubules seem to be more easily broken than the flagella (Figs 18, 19) In general microtubules are found together with membrane fragments, but it has also been observed that some lie free on the supporting film of the grid (Fig 19) The structure of cells suspended in 5×10^{-4} M EDTA in $2 5 \times 10^{-3}$ M TRIS buffer pH 9 0 did not differ from the structure of well preserved cells suspended in SMC

Observations on Sectioned Material

In sections of glutaraldehyde osmium tetroxide fixed organisms the treponemes show an outer three layered membrane, an intermediate layer which appears to adhere to the outer layer of the cytoplasmic membrane

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All illustrations show material from *Treponema* Reiter Unless otherwise stated the bar on each micrograph represents 0 1 μm

Fig 1 Organism treated on the grid with Teepol for 30 seconds Note the characteristic spotted appearance of the cytoplasm Some flagella (F) wind around the organism though their basal discs (arrows) are no longer inserted into the cytoplasm Negatively stained with 1 per cent ammonium molybdate Magnification 80 000 ×

Figs 2–3 Endoflagella liberated after treatment as for Fig 1 A narrow collar (C) connects the hook (H) to the basal disc (B) Note the substructure of the hook which differs from that of the shaft Membrane fragments adhere to the basal disc in Fig 2 but not to that of Fig 3 Negatively stained with 1 per cent ammonium molybdate Magnification 160 000 ×

Figs 4–5 Endoflagella liberated after treatment of *suspended cells with sodium deoxycholate* Each basal disc (B) consists of a single disc shaped plate The substructure of the collar (C) differs from that of the hook (H) Negatively stained with 1 per cent lithium tungstate Magnification 160 000 ×

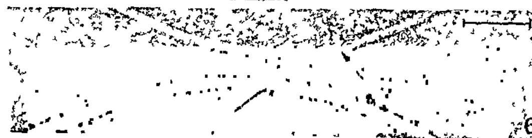
Fig 6 A flagellum (arrow) which has lost the sheath after repeated washings with redistilled water Flagella were freed after sodium deoxycholate treatment A sheathed flagellum (F) is seen in the same field of view Negatively stained with 1 per cent ammonium molybdate Magnification 160 000 ×


F
1


B
C
H
2
B
3


B
C
H
4


B
C
H
5


6

and a three layered cytoplasmic membrane (Fig 20) The flagella are seen between the cell wall and the intermediate layer (Fig 20) The ribosomes are rather evenly distributed in the cytoplasm The nuclear region can be distinguished as less dense areas containing delicate strands (Fig 21) The whorled type of mesosome has been seen and occasionally a continuity with the plasma membrane could be resolved Sometimes there is a tendency for the cytoplasm to be less dense in the area where the microtubules are situated This enables these to be recognized in the cytoplasm of some cells The microtubules are, when possible to depict seen in the cytoplasm close to the inner layer of the cytoplasmic membrane (Fig 20) The bundle of microtubules is situated immediately below the bundle of flagella (Fig 20) and the diameter of the individual microtubules is about 75 Å

The organisms which were fixed with glutaraldehyde alone, were not as well preserved as those fixed by the combined glutaraldehyde osmium tetroxide method The overall contrast was extremely low in sections observed without any poststaining However, staining with a 1 10 dilution of the standard lead citrate gave good contrast on all membranes including the few mesosomes present as well as flagella and microtubules, while no considerable increase in ribosomal contrast and cytoplasmic density was observed The flagella do not seem to be as well preserved as after the double fixation but are recognizable between the cell wall and the plasma membrane (Fig 22) The microtubules however, seem to be well preserved and are quite distinct in the cytoplasm close to the inner layer of the plasma membrane (Fig 23)

DISCUSSION

The detergent Teepol acts on the organisms by destroying the membranes of the cell wall The cell wall is not visible in sections of cells embedded after Teepol treatment whereas the plasma membrane is still evident The flagella are easily detached by treatment with dilute Teepol This probably means that the plasma membrane is somewhat altered or weakened by this treatment, at least around the insertion point However, remnants of membranes are often seen on the basal end of the isolated flagella This could mean that the flagella are torn out of the insertion region as a result of the surface tension forces acting during drying of the specimen On the other hand, fewer flagella are liberated after treatment with deoxycholate and the basal discs of such flagella are less obscured by membrane fragments than those detached following Teepol treatment Obviously, the effect on the plasma membrane in the insertion region differs for these two detergents

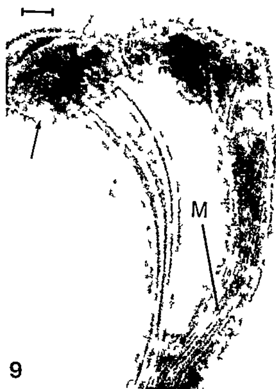
A sheath was found to cover only the shaft of the flagellum proper and was never seen to extend to any part of the hook at the proximal end The approximate thickness of the sheath of the flagella is about 30 Å a figure arrived at by measuring the difference in over all width between sheathed and unsheathed flagella *Lowy* & *Hanson* (15) estimated the thickness of the sheath of the flagella of *Pseudomonas rhodos* in this way and found the sheath to be 25 Å thick The approximate thickness of the coat of isolated axial filaments of *Leptospira* strain B16 can be calculated from the observations of *Nauman et al* (17) to be about 35–45 Å At present it seems impossible to judge from the limited number of observations published whether the small differences in sheath thickness found on flagella from different micro

Figs 7–8 Parts of organisms treated on the grid with sodium deoxycholate for 2 minutes Note the bundles of microtubules (M) which wind around in the interior of the cells The helical outline of the treponemes is quite distorted Negatively stained with 1 per cent ammonium molybdate Magnification 90 000 ×

Fig 9 Part of an organism treated as for Fig 7 Some flagella are inserted in remnants of the cell (arrow) A bundle of microtubules (M) is also present Negatively stained with 1 per cent ammonium molybdate Magnification 90 000 ×

M





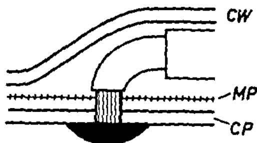
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organisms reflect true differences or are a result of variations in technique

The hook differs from the flagellum both in width and in substructure The diameter of the hook is slightly greater than the diameter of the unsheathed flagellum This is in accordance with the observations of *Abram et al* on flagella isolated from *Proteus vulgaris* (1), and with the observations of *Cohen Bazire* & *London* on flagella from three species of *Rhodospirillum* (5) For flagella isolated from *Bacillus spp* *Abram et al* (2) reported that the diameter of the hook was slightly less than the diameter of the flagella *Holt* & *Canale Parola* (9) measured the diameter of unsheathed filaments from *Spirochaeta stenostrepta* as 9 10 nm, and the diameter of the hook to be of approximately the same order The difference observed in the substructure of the flagellum and the hook has been noted on nearly all flagella isolated from bacteria both gram positive (2) and gram negative (1, 5 8 14) and has also been observed on filaments isolated from *Spirochaeta stenostrepta* (9) and from *Leptospira* (17)

The honeycombed structure of the hook has been found in isolated flagella studied in the papers mentioned above (1, 2 5 8, 9 14 17) and a narrow collar between the hook and the basal disc has been reported by *Cohen Bazire* & *London* (5) for *Rhodospirillum spp* and by *Abram et al* (2) for *Bacillus spp* The narrow collar can also be seen on the isolated filaments from *Sp stenostrepta* (9)

We have been unable to find more than a single disc at the insertion end of flagella isolated by any of the methods used *Holt* & *Canale Parola* (9) were also unable to find two well-defined paired discs at the basal end of the filaments they isolated from *Sp stenostrepta* According to these authors the method they used for obtaining suspensions of free filaments might have damaged the basal discs and the existence of well defined paired discs was more or less inferred The AL-1 enzyme treatment we used for isolation of flagella from the treponemes was identical to that used by *Cohen Bazire* & *London* (5) for their study on flagella from species of *Rhodospirillum* In their material two paired discs at the basal end of the flagella were clearly present In this laboratory we have also used the same method for resolving the basal end of axial filaments from *Leptospira pomona* (4) We found two discs in this material thus confirming the similar observation of *Nauman et al* (17) on another species of *Leptospira* Consequently we tend



Text Fig 1

The sheated shaft and the hook proper of an endoflagellum is situated underneath the cell wall (CW) and outside the cytoplasmic membrane (CP) The narrow collar penetrates the intermediate or mucopeptide layer (MP) as well as the cytoplasmic membrane, and thus connects the basal disc to the hook The basal disc is situated in the cytoplasm close to the inner layer of the cytoplasmic membrane

Fig 10 Part of an organism after treatment on the grid for 1 minute with AL-1 enzyme Only flagella (F) bundles of microtubules (M) and some membraneous debris (arrows) are left Negatively stained with 1 per cent uranyl formate Magnification 90 000 ×

Fig 11 A tuft of 8 flagella attached to a membrane fragment Specimen prepared from pellet of material from suspended cells after treatment with AL-1 enzyme Negatively stained with 1 per cent ammonium molybdate Magnification 160 000 ×

Figs 12 13 Endoflagella liberated by the same treatment as for Fig 11 The basal discs (B) are attached to membrane fragments Each basal disc consists of a single plate The collars (C) between the basal discs and the hooks (H) are clearly seen Negatively stained with 1 per cent ammonium molybdate Magnification 160 000 ×

F
M
F
10


11


B
C
H
12


B
C
H
13

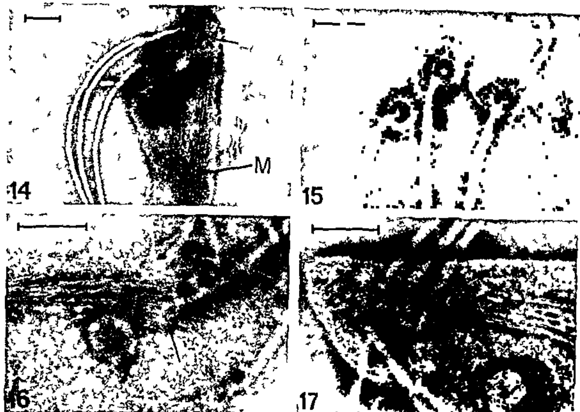


Fig 14 Part of an organism treated on the grid with AL-1 enzyme for 30 seconds A bundle of 6–8 microtubules (M) is seen Note the rings around the basal discs of the flagella (arrows) Negatively stained with 1 per cent ammonium molybdate Magnification 80 000 ×

Fig 15 Some flagella liberated after treatment on the grid with AL-1 enzyme for 4 minutes Ringshaped structures (arrows) are seen around the basal discs of the flagella Negatively stained with 1 per cent ammonium molybdate Magnification 160 000 ×

Figs 16 17 Ends of cells after treatment on grid with AL 1 enzyme for 1 minute The ends of the microtubules are seen close to the insertion points of the flagella (arrows) Negatively stained Fig *16* with 1 per cent ammonium molybdate Fig 17 with 1 per cent lithium tungstate Magnification 160,000 ×

to believe that our finding of a single disc at the basal end of the endoflagella of *Treponema* Reiter presents the true picture, and our interpretation of how the flagella are inserted into the cytoplasmic body of the organism is illustrated in the schematic drawing of text Fig 1 All our observations are not accounted for in this figure, for example the ring around the basal disc in preparations after AL 1 enzyme treatment (see Figs 14, 15) is not included Such a ring could be a reinforcement of the cytoplasmic membrane around the insertion point or it could arise from accumulation of heavy metals in an indentation of the membrane around the basal disc

Two bundles of microtubules, one in each end of the cell, were observed in cells treated with sodium deoxycholate or AL 1 enzyme The diameter of each microtubule was about 75 Å both in negatively stained preparations of treated cells and in thin sections of cells fixed and dehydrated according to the method of *Hills* & *Plasskitt* (7) The number of microtubules in the central region of the cell is about twice the number of microtubules present at each end Suitable cross sections of embedded cells show these bundles





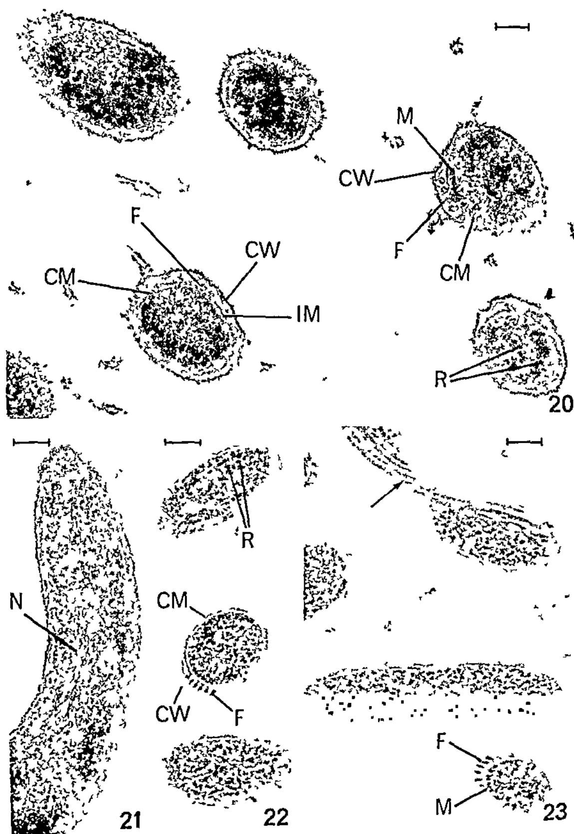
18 M crotubules in the m ddle of a cell treated as for Figs 16 17 The electron dense stain penetrated into the lumen of the tubules (arrows) The microtubules appear broken Some flagella) are also present Negat vely sta ned w th 1 per cent ammonium molybdate Magnif cat on) 000 ×

19 Specimen prepared from pellet of material from suspended cells after treatment with AL 1 en ne The microtubules (M) seem to be more easily broken th an the flagella (F) Some m crotubules lying free on the supporting film (arrow) Negatively sta ned w th 1 per cent ammon um molyb Magnificat on 160 000 ×

microtubules to be situated in the cyto sm in close contact with the inner leaflet the cytoplasmic membrane and always mediately underneath the bundle of flag

Usually 1–3 more microtubules than gella are present in a cross section of an anism These observations corroborate ose of *Klingmuller et al* (12) on their dy of *Treponema pallidum* Nichols From atively stained material it can be deduced at the microtubules wind around in the erior of the cell in the same way as the gella do in the interspace between the l wall and the cytoplasmic body of the anism Until now we have failed to pre it convincing evidence for any direct connection between the basal discs of the flagella and the microtubules The apical ends of the microtubules are generally seen very close to the basal discs of the flagella and the microtubules have certainly never been observed to extend beyond the zone of insertion for the flagella

Treatment with Teepol apparently destroys or intensively damages the intracytoplasmic microtubules Neither in sectioned cells nor in negatively stained material from cells subjected to this treatment has it been possible to find these microtubules After the same treatment flagella are always present This indicates that the chemical constituents of microtubules and flagella are not identical


M
CW
F
CM
F
CW
CM
IM
R
20
R
N
CM
CW
F
21
22
F
M
23

otubules might still, however, function
e site of synthesis for the flagellar pro-
or its precursors Another possibility is
they may serve as a kind of cytoskeleton
athway for intracytoplasmic transport for
eiter treponemes in much the same way
microtubules of 175–275 Å in dia-
r, present in animal and plant cells (3,
3), are thought to do

present any suggestion, however, for
ossible functional role of the intracyto-
nic microtubules in *Treponema* Reiter
be highly speculative Isolation and
ication of sufficient amounts of these
lles to carry out chemical analysis, in
treatment with various enzymes are
usly needed, in order to obtain informa-
their chemical constituents Such in-
ation might lead to a better understand
their function.

authors wish to thank Mr *F Laursen*, Mrs
and Mrs *B Skeel Christensen* for their
nt assistance in electron microscopy and
ing and Miss *A G Overgaard* and Mr
sen for their expert photographic work We
s our gratitude towards Dr *H Aa Niel*
reponematoses Department, Statens Serum

Transverse sections of cells after glutaralde
mium tetroxide fixation The flagella (F)
between the cell wall (CW) and the
smic membrane (CM) The intermediate
IM) adheres to the outer leaflet of the
smic membrane In the cytoplasm ribo-
(R) are distributed Microtubules (M) can
cted right under the bundle of flagella
cation 90 000 ×

Section illustrating a nuclear region with
strands of nuclear material (N) Same
as for Fig 20 Magnification 90 000 ×

Section of organisms fixed in glutaralde
ly and dehydrated as stated in text Flag
) are seen between cell wall (CW) and
ic membrane (CM) Ribosomes (R) are
the cytoplasm Magnification 90,000 ×

Section from the same preparation as
in Fig 22 A bundle of microtubules (M)
is close to the inner layer of the cyto-
membrane and immediately below the
flagella (F) The arrow points to longi
y sectioned flagella Magnification 90,000 ×

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THE FINE STRUCTURE OF *CARDIOBACTERIUM HOMINIS**

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Three strains of *Cardiobacterium hominis* (31) were studied with the electron microscope Thin sections showed slender rods with a cell wall of the coherent Gram negative type and with a cytoplasm containing numerous intrusive membranes disposed around the periphery of the cells especially at the poles The cell wall consisted of a unit membrane sandwiched between dense outer and inner layers, the outer dense layer showing a 4-5 nm thick regular structure Polar caps of a dense tufty material were noted on the ends of cells The sub structure of the surface layer was studied in negatively stained cell wall preparations ob tained after ultrasonic treatment It consisted of a close packing of nearly spherical units (averaging 3 5 nm in diameter and with a repeat frequency of 5 5 nm) in a rectangular array it was also demonstrated after freeze-etching This layer represents an unusual type of surface structure on a Gram negative bacterium

964 *Slotnick* & *Dougherty* (31) named described a new bacterial genus and spe- *Cardiobacterium hominis*, which was named because it was originally isolated human endocarditis, and incorporated strains previously designated as "Group) (32, 36) *Snyder* & *Ellner* (33) and *lue et al* (22) described one case each ndocarditis caused by *C hominis* Clini cases have been infrequent The organism be found as part of the flora of the upper respiratory tract in many normal individuals and it is seldom isolated from other regions of the body (30, 32) Recently *Midgley et al* (12) isolated *Cardiobacterium hominis* from two patients with bacterial endocarditis and they studied these strains in detail together with the strains (NCTC 10426 and 10427) isolated by *Slotnick et al* (32), which we have included in our study *Hill* & *Lapage* (9) compared *Bacteroides corrodens* and *C hominis* and concluded that neither of them possessed characters of any of the recognized genera of the family *Brucellaceae*, nor did they resemble each other sufficiently to be classified as two species of one and the same genus The G + C content of their DNA was about 57 and 62 moles per cent, respectively

We chose to study *Cardiobacterium hominis* because it was reported by *Slotnick* & *Dougherty* (31) to be Gram negative "although Gram positive stain may be retained

Received 30 vii 70

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* Presented in part before the Second Meeting of the North Western European Microbiological Group Stockholm, June 16-18, 1969 (Reyn, Murray, Birch Andersen 1969)

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in swollen ends or central portions of cells' It had been observed (24, 25) that "*Neisseria haemolysans* (34) or "*Gemella haemolysans* (1), hitherto described as being Gram negative or Gram variable, possessed a Gram positive type of cell wall in addition to other features indicating an incorrect assignment to the family *Neisseriaceae* Thus it was thought that the anomalous Gram reactions reported would be reflected in the fine structure of *C hominis* and the results might be of taxonomic use Although the premise was not realized, the present paper reports that *C hominis* possesses some unusual structural features revealed by the electron microscope

MATERIALS AND METHODS

Three strains were examined NCTC 10426 and NCTC 10427 were obtained from the National Collection of Type Cultures London England and SS 1742/1966 was obtained from the Diagnostic Bacteriology Department Statens Serum institut, Copenhagen The organism was repeatedly isolated from the blood of a 28 year-old man with endocarditis The bacteriological findings were in agreement with those reported by *Midgley et al* (12) They were all definitely Gram negative according to the methods used in this laboratory (25 26)

Two media were used (1) Haemoglobin yeast liver agar (18) with 6 7 per cent heated horse blood instead of haemoglobin (modified HYL medium) and (2) yeast extract sodium acetate peptone medium 0 3 per cent 0 05 per cent and 0 3 per cent respectively of the Difco products (YAP)

Cultures were harvested by washing cells off the agar with YAP broth or 0 2 per cent osmic acid in 1 + 4 diluted barbiturate acetate buffer (28) after 18 20 hours incubation on HYL agar at 36° C in a moist 10 per cent CO atmosphere

Preparation for embedding and sectioning Prefixation was attained by adding osmic acid (1 per cent osmic acid in barbiturate acetate buffer pH 6 1) as prescribed by *Ryter & Kellenberger* (28) to freshly made suspensions in YAP broth to give 0 1 per cent final concentration alternatively 1/10 volume of 12 5 *per cent glutaraldehyde* in the same buffer was added After 30–60 minutes at room temperature the cell suspensions were centrifuged at 2500 *g* for 10 minutes and the resulting pellets enrobed in agar at 45° C (1 5 per cent agar in barbiturate acetate buffer at pH 6 1) Agar blocks of about 1 mm³ were cut and fixed over night at room temperature in 1 per cent buffered OsO_4 to which was added 0 1 per cent YAP broth After a brief wash in barbiturate buffer the agar blocks were post treated for 1 hour in 2 per cent uranyl acetate in barbiturate buffer they were then acetone dehydrated and finally embedded in Vestopal W (29) After hardening of the plastic sections were obtained on the LKB ultrotome I and/or III microtomes Double staining of sections was performed by floating grids for 15 minutes on a 7 per cent solution of magnesium uranyl acetate (7) followed by a 2 minute treatment with lead citrate (27)

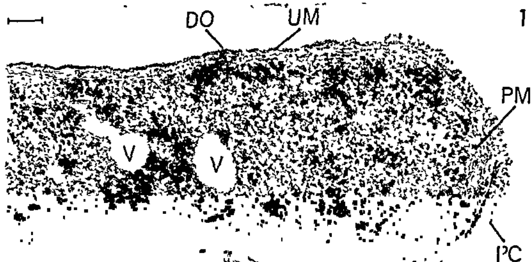
Negatively stained preparations Cell suspensions in 0 01 M calcium chloride were obtained from 18 hour and 5 day old cultures grown on modified HYL agar The cells were broken up by supersonic treatment (30 120 seconds), while cooled in crushed ice, using either an Elma watch cleaning apparatus or an MSE 500 ultrasonic disintegrator to obtain a 25 30 per cent drop in turbidity Whole cells were removed at 5000 *g* in 5–7 minutes and the suspended wall fragments were spun out at 20 000 *g* for 12 minutes Negatively stained preparations (3) of the three strains were made from the sediment suspended in a small volume of water using a platinum loop method (14) No addition of colloidal substances to encourage spreading of the material on carbon reinforced formvar films was necessary but the grids were exposed to a UV source at about 10 cm range for 15 minutes to make the surface charge more suitable The negative stains used were 1 per cent ammonium molybdate adjusted to pH 7 with NH_4OH and 1 per cent uranyl formate at pH 4 5 5 0 (*i e* unbuffered) The results obtained with these were without any significant differences

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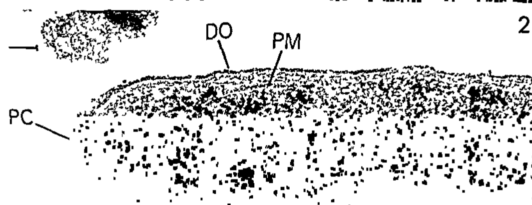
Unless otherwise stated the bar on each micrograph represents 0 1 µm The following abbreviations are used PC – polar cap UM trilaminar unit membrane component of cell wall DO = dense outer layer of cell wall DI – dense inner layer of cell wall CM – cytoplasmic membrane V = vesicles PM = paired membranes RS = regular surface structure

Fig 1 NCTC 10426 Note trilaminar unit membrane component of wall dense outer layer polar cap vesicles and paired membranes beneath polar cap Inset Part of a lysed cell showing outer dense layer and unit membrane of cell wall as well as cytoplasmic membrane Magnification 90 000 ×

Fig 2 NCTC 10426 Note dense outer layer and dense inner layer of cell wall polar cap and paired membranes Magnification 90 000 ×


DO
UM
1
PM
V
V
PC


DO
UM
CM
DO


2
DO
PM
PC


DO
DO
DI
DI

Microscopy Sections as well as negatively stained preparations were examined with a Philips EM 200 or EM 300 electron microscope Exposures were made on Eastman Kodak fine grain release positive film type 5302 at primary magnifications of about 9000, 17000 or 35000 × and suitable fields were photographically enlarged as desired

Free e etching Cell suspensions obtained as be fore and in 0 01 M calcium chloride were cen trifuged at 5000 *g* The pelleted cells were placed in small drops on 3 mm specimen holders frozen in Freon 22 stored in liquid nitrogen cleaved and etched by standard methods (13) in a Balzers Unit (Model BA 510 M), shadowed and repli cated with platinum carbon supported with car bon repl cas cleaned with successive treatment with 70 per cent sulfuric acid and with hypo chlorite and washed with distilled water before picking up on 200 mesh grids

RESULTS

The cells of *C hominis* were small Gram negative rods and looked quite regular in the light microscope Sections of the embedded cells (Figs 1–4) showed somewhat wrinkled profiles, as if the fixation method was not entirely suitable although the size relations were only slightly reduced (0 3–0 4 × 1 2–2 μm) The cell wall was of the Gram nega tive type with coherent layers (8 16 19), the trilaminar unit membrane component of the wall remained parallel to the innermost dense layer and the zone between them was of intermediate density (Figs 1 and 2)

A prominent feature of all three strains was a polar cap of material 20–40 nm thick adhering to the outside of the cell wall and confined to the terminal portion of the cell (Figs 1–4) hardly extending to the sides of the cylindrical cell The section profile of the cap gave the impression of a radial arrange ment of tufts of material, the material took up the metal stains quite avidly

A feature of the cell wall profile was a 4–5 nm thick dense layer on the outer surface which was most clearly seen along the sides because it was obscured by the polar cap on the ends of the cells (Fig 2 and inset Fig 1) This dense outer layer gave the impression of a very fine repeating structure (Fig 2) and was closely applied to the outer lamina of the unit membrane component of the wall giving an unusually dense and coherent look to this otherwise conventional Gram negative wall The total thickness of the cell wall was 20–22 nm

The cytoplasm enclosed in a plasma mem brane of usual dimensions (ca 8 nm), was not remarkable except for a profusion of unit membrane profiles of similar dimensions and presumed to be intrusions of the plasma membrane Some sections showed (more in NCTC 10426 than in the other two strains) that they were peripherally distributed ir regularly folded and roughly parallel to the surface (Fig 1), which made it difficult to distinguish the plasma membrane in many places, other sections exhibited more exten sive intrusions of paired membranes towards the centre of the cell which produced con siderable areas apparently free of ribosomes when tangential sections of these membranes were obtained (Fig 4) Identification of membrane pairs was not easy and in one case (Fig 1) pairs of membranes seemed to include ribosome like structures Most pairs (*e g* Fig 2) showed an intermediate density and no ribosomes between them

Some preparations (Fig 1) showed mem brane lined vesicles apparently empty, with out clear continuity with the other intruded membranes Peculiar profiles were observed in some sections going through the peripheral zone at a shallow angle and nearly tangential to the surface presumably because there are areas (see Figs 3 and 4) of unusual con volutions of membrane and distribution of contents Ribosomes were plentiful in the cytoplasmic matrix and the nucleoplasm was diffusely scattered throughout the cytoplasm (Figs 1–4)

Negative staining revealed a cell wall sub structure and the margins of intact cells showed that this was the superficial layer (Fig 5) and thus corresponding to the dense

Figs 3 and 4 NCTC 10427 and SS 1742 Note areas with convoluted membranes and peculiar profiles of cells sectioned at shallow angle to the surface Magnification 45 000 ×

0.5 μm

3

0.5 μm

4

5

RS

RS

6

RS

RS

RS

er layer seen in sections (Figs 1 and 2), cell wall fragments obtained from soni ed suspensions allowed more effective reso on (Figs 6–9) The structure was the ie for the three strains and consequently illustrative material derives mainly from of them SS 1742

The regular surface structure (RS) con of units packed in a rectangular array ctically all the fragments were folded to varying degrees of moire interference terns and areas identifiable as single layers e unusual (Fig 9) Optical diffraction ved no hint of other than a two-fold metry although some distortion of the y in isolated areas was evidently possible 9)

he repeat frequency was easily measured ig the edges of folded sheets of units s 6–9) the average measurement of tre to centre spacing for both axes proved e 5 5 nm The average diameter of the was 3 5 nm and thus the space between approximated to 2 nm No effective lution of unit structure was obtained but appeared to be nearly spherical (Figs d 9)

eeze-etching provided definite evidence the RS component was on the outer ce of the wall The resolution of struc was imperfect and only one dimension e array was shown by shadowing when as almost normal to the plane of that nsion The angle of shadowing had little t judging by comparison of the details a curved surface Only one parameter be measured and the ridge to ridge ng averaged 4 6 nm which was about r cent of the measurements of RS made egatively stained preparations Cleaved freeze-etched cells revealed no useful new information on the disposition of mem branes

Neither negative staining (whole cells or cell wall fragments) nor freeze etching pre parations showed any recognizable compo nents of the polar caps

DISCUSSION

The observation that *C hominis* tended to retain the Gram complex (31) has not been confirmed and there is no doubt that the section profile of the cell wall is directly comparable to the type exhibited by Gram negative bacteria (8) There may have been some changes in the morphology exhibited by cultures serially transferred on rich media and it is our impression shared by *Midgley et al* (12) that the cultures are now Gram negative and only slightly pleomorphic However, both studies included freshly isolated strains which appeared to be entirely com parable to the stock cultures

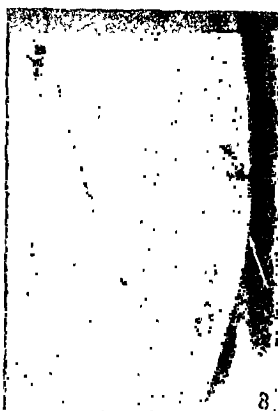
The polar caps are a consistent feature of sections of the strains studied and probably represent a particularly localized synthesis at the sites of a previous division One can only guess at the composition Bacterial capsules that persist through the preparation proce dure for sectioning and take up stains such as uranyl acetate are generally polypeptide in nature We have seen a similar tendency to radial arrangement in the microcapsule of *Branhamella catarrhalis* (4) (unpublished observations) A localized arrangement of components at the poles and equatorially was identified using immunological methods and light microscopy by *Tomcsik* (35) in the capsule of *Bacillus megaterium* In the pre sent example no accretions similar to the polar caps were seen at the sites of a forth coming division so we must assume that this special synthesis and local accumulation oc curs later after division has been completed

The RS layer is unusual because of the tetragonal or rectangular packing of the units and the fine repeat frequency of the array on the wall of a Gram negative bacterium In their recent review of bacterial ...

SS 1742 Note regular structure of super layer at marg n of intact cell Negat ve sta n cent ammon um molybdate pH 6 5 Mag on 170 000 ×

SS 1742 Note regular structure of s per layer Negative stain 1 per cent uranyl te pH 4 5–5 0 Magnif cat on 170 000 ×





9

hly *Glauert & Thornley* (8) identified one tetragonal array on a Gram nega- (an unpublished study of their own on *Acinetobacter*) However, an extremely lar structure "with a periodicity of 40Å" observed by *Remsen et al* (23) in meme fractions of *Nitrosocystis oceanus* dised in a French pressure cell At that time thought that this structure belonged to of the cytomembranes of this membraneed organism Recently *Watson & Rem-* (37) have found that the tetragonal y "of 40 A particles" (the periodicity aps to be about 7 5 nm) is a part of the wall and lies *underneath* two other strucl wall components (one of them a comhexagonal array with a periodicity of 12 and is itself very close to or a part of double track layer No such anatomical usion exists for *C hominis*, the RS array on the outside of the double track layer ction profile and can be peeled from it in ing for freeze etching

am positive bacteria have more often n rectangular arrays on their surfaces 0 11, 20) and these bacteria are, so far, cted to *Bacillus* and *Clostridium* species units generally show a periodicity of oximately 10 nm In the case of *B polyy* it has been shown by optical diffraction integration methods (6, 21) that each consists of four sub-units (each about m) closely set together to form a tetrad is not easily resolved But when the is first formed, during spore germina the individual sub-units are evenly seed so that the array has an evident dicity of a little more than 5 nm (15) nstancy of the periodicity in the RS is remarkable but views of considerable

and 8 NCTC 10427 and SS 1742 Note and spacing between units of the regular structure (arrows) Negative stain 1 per mmonium molybdate pH 6 5 Magnification 00 ×

SS 1742 Note single layer of regular structure (arrow) Negative stain 1 per uranyl formate pH 4 5 5 0 Magnification 00 ×

areas of cell surface show a mosaic of orientations (see Fig 5), which is not unusual among RS layers (2, 10) The units and their spacing are on as small a scale as any natural array shown up to now and it seems probable that each unit represents a single macromolecule There is no direct chemical evidence of its nature An analogous structure, *e g* that of *B polymyxa*, appears to be made of a protein (20)

The internal organization of membranes has not received more than incidental study A similar disposition of intrusive membranous laminae is reminiscent of the arrangement in *Nitrosomonas* (17) in that they are mainly peripheral, concentrically arranged and membrane pairs are not in any rigid relationship

The authors wish to thank Dr *S P Lapage* Curator, National Collection of Type Cultures, Central Public Health Laboratory, London, England for suggesting the study of the fine structure of *Cardiobacterium hominis* and for providing two cultures of this organism Dr *W Frederiksen* and Dr *P Bulow* of the Diagnostic Bacteriology Department, Statens Seruminstitut, Copenhagen, are thanked for providing a third culture Dr *R G E Murray* is grateful for personal support by the Medical Research Council of Canada as a Visiting Scientist 1968–69

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HAEMOLYTIC ACTIVITY OF *ACINETOBACTER CALCOACETICUS*

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The haemolytic activity of broth culture filtrate of *Acinetobacter calcoaceticus* grown at 22°C has been investigated The haemolytic activity was enhanced by Mg^{++} and Mn^{++} and inhibited by Zn^{++} and Hg^{++} Red cells from different species exhibited different haemolytic patterns The temperature coefficient ($Q_{10°C}$) was estimated at 1 8, the reaction being rectilinear for 1 hr at 37°C The haemolytic material was non dialysable, sensitive to heat and proteolytic enzymes and antigenic on injections of filtrate into rabbits The nature of the haemolytic material is briefly discussed

netobacter calcoaceticus (1) is now widely epted as the species name of strains nerly classified as e g *Micrococcus calcoticus, Bacterium anitratum, Alcaligenes molysans, Achromobacter anitratus* and *netobacter anitratus*

I *calcoaceticus* is frequently found as a taminant in bacteriological specimens n human sources For this reason, its clini significance has been a matter of discus- ı Potent endotoxic substances have been ionstrated in *A calcoaceticus* (4) An in- ısing number of clinical reports (2,5) gests its pathogenicity, especially in in ıduals with lowered resistance to bacterial ctions

Haemolysis on blood agar is a common ling in strains of *A calcoaceticus*, and has n used for purposes of classification (8) nriksen (1937) investigated haemolytic ıins of *Alcaligenes* isolated from the resp ory tract, and demonstrated a heat labile, rable haemolysin in broth cultures The purpose of this paper is to describe some observations of the haemolytic activity of a strain of *A calcoaceticus*

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MATERIALS AND METHODS

The strain of *A calcoaceticus* studied (1318/69) was isolated from the urine of a 70 year-old male patient The organism was grown on blood agar plates with 6 per cent human blood It was kept freeze-dried or transferred weekly on broth (beef extract 1000 ml, peptone Witte 10 g, NaCl 5 g, pH 7 6) This medium was also employed for the production of haemolytic crude filtrates Unless otherwise specified, incubation temperature was 37°C for solid cultures and 22°C for liquid cultures The organism was a non motile, coccoid, at times diplobacillar, Gram negative rod Growth was obligate aerobic and occurred on most of the commonly employed culture media On blood agar, the colonies were yellowish gray, with a butyrous surface and a sticky consistency The colonies were surrounded by a broad zone of haemolysis

On a bromthymol-blue agar plate with lactose 10 g/l, the organism revealed no acid production in 24 hrs, but if the lactose concentration was raised to 100 g/l, or the incubation period extended to 48 hrs, acid production became visible No growth occurred at 4°C or 45°C, but growth occurred readily between 20°C and 37°C When broth cultures were incubated at 37°C, a thread like precipitate was formed in 48 hrs

Acid production from carbohydrates was examined on a broth medium, containing 1 per cent carbohydrate, adjusted to pH 7,6, and incubated at 37°C. Acid was produced from glucose, xylose, arabinose, galactose, and mannose. Eighteen other carbohydrates, including lactose and starch, did not reveal acid production. The organism was oxidase negative and did not reduce nitrate. Gelatin was not liquefied, a blood plasma coagulum was slowly liquefied. It was sensitive to streptomycin, tetracyclin, neomycin and sulphonamides, but resistant to penicillins, erythromycin, chloramphenicol, cephalosporin and polymyxin B, as examined according to the method of *Ericsson* (3).

Preliminary observations of the haemolytic activity were made by transferring haemolytic material (agar or liquid media) to the surface of intact blood agar plates, and observing early visible haemolysis. Subsequent investigations were performed by means of human red cell suspensions, except in one experiment specified below. Blood from a healthy adult male (type A, Rh+), was drawn every 2–3 weeks into equal volumes of Alsever's glucose citrate solution adjusted to pH 6,1 and kept at 4°C until use. Cell suspensions were prepared daily by washing twice with 10–20 vol. of 0,145 M NaCl, and then packing by centrifuging at 1 300 *g* for 10 min. A cell suspension of 1 per cent was employed, diluted in 0.1 M tris buffer, pH 7.6, containing 0,130 M NaCl and 0,010 M $MgCl_2$.

Determination of haemolytic activity. Samples to be assayed were serially diluted in twofold steps in 0,145 M NaCl. To 0.5 ml portions was added 0.5 ml red cell suspension. The tubes were incubated for 60 min at 37°C, with frequent shaking to prevent sedimentation, then cooled for 2 min in ice-water and finally 2 ml 0.145 M NaCl were added to each tube. After centrifuging for 10 min to remove unlysed cells, optical density in the supernatant was read in a Linson jr photometer at 540 nm in a flowthrough cell with 10 mm light path, 30–120 min after the end of the incubation period. Blanks showed negligible values, but were always included. One haemolytic unit (H.U.) is defined as the amount of haemolysin that will produce 50 per cent haemolysis of 1 ml 1 per cent human red cell suspension by the method employed. The calibration graph seen in fig. 1 was based on assays in duplicate and showed linearity between 25 and 70 per cent haemolysis. The precision of the method was examined in 10 replicate series of 5 dilution steps. The standard deviation ($s = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$), expressed as per cent of the mean (coefficient of variation c.v. $= \frac{100 \cdot s}{\bar{x}}$) was 4,3.

Preparation of crude filtrate. The broth medium was inoculated with 1/200 vol. of a 24 hrs broth culture of *A. calcoaceticus* and incubated aerobically at 22°C. The growth rate was estimated turbidimetrically in a Linson III photometer at 620 nm. After 72–96 hrs, the culture was centrifuged at 10 000 *g* in a Servall Superspeed centrifuge at 4°C and filtered through a Seitz filter. The filtrate was kept at –20°C. Haemolytic activity ranged between 35 and 60 H.U./ml and remained unchanged at –20°C for 3 months. Protein concentration was estimated at 10 mg/ml by the biuret reaction (9). Dialysis was performed in dialysis tubes 8/100 FT (Union Carbide Corp., Chicago, Ill.) for 24 hrs at 4°C against 200 vol. 0,145 M NaCl. Heat inactivation was investigated by heating undiluted crude filtrate in water baths (periods and temperatures specified below), followed by rapid cooling in ice water and determination of remaining haemolytic activity. Degradation by enzymes was examined with Crystalline Trypsin (Novo, batch No 60218), Crystalline α Chymotrypsine (Novo, batch No 27), Pronase (Calbiochem B grade, lot 54909) and Papain (Sigma 2 × crystallized lot 97 B 2430–1). Blanks were included to permit estimation of the spontaneous decrease in haemolytic activity during the incubation period, and correction for possible haemolysis caused by the enzymes added.

Six month-old rabbits were immunized by repeated injections of 0.5 ml crude filtrate. The antihaemolytic titre was calculated on the basis of 50 per cent inhibition of haemolysis.

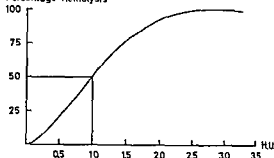
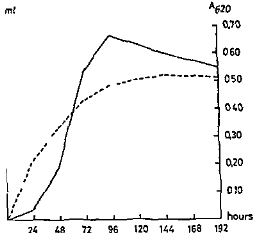


Fig. 1. Calibration Graph for Haemolytic Activity. A dilution of filtrate to produce appr. 50 per cent haemolysis was roughly estimated, and used as a basis for calculation of dilutions to produce from a few per cent to complete haemolysis. The point of 50 per cent haemolysis was found by means of a 50 per cent haemolysis standard, and the corresponding amount of haemolytic material was fixed as one haemolytic unit, by the method described.

RESULTS

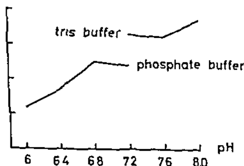
Preliminary observations on solid media revealed that visible haemolysis and visible



2 *Growth Curve and Haemolytic Activity*
Haemolytic activity in crude filtrate from broth ture, according to the test method — Turbidity the culture read in a Linson III photometer at) nm

›wth occurred simultaneously and that emolytic material was produced in the ab ıce of red cells On blood agar plates devoid peptone, a brown discolouration developed ıtrally in the haemolysis zone Transfer of adial section of the haemolysis zone to the face of intact blood agar revealed that the molysin had been inactivated in the dis oured area Discolouration and inactivation

e of 50%
nolysis



3 *Haemolytic Activity at Different pH Values*
the pH range 6–7 2 a 0 03 M phosphate buffer ıtaining 0 080 M NaCl and 0 010 M $MgCl_2$ used In the pH range 7 2–8 a 0 1 M tris buffer ıtaining 0 130 M NaCl and 0 010 M $MgCl_2$ was d. Both series were examined according to the t method

were less pronounced at 22°C than at 37°C, and in the presence of peptone Efforts to produce haemolytic crude filtrates from broth, a casein hydrolysate medium and from peptone water at 37°C were not successful When the incubation temperature was lowered to 22°C, activity occurred in the broth culture The growth curve and haemolytic activity in the broth filtrate are seen in Fig 2

Human and rabbit red cells behaved in the same manner Sheep red cells were not haemolyzed during the incubation period (37°C), but haemolysis developed during the subsequent cooling in ice water (hot cold effect) The titre was lower than in the human and rabbit red cell series Human red

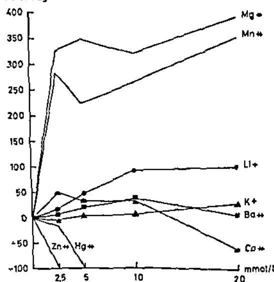


Fig 4 Activation and Inhibition of Haemolytic Activity by Various Cations
Crude filtrate dialysed against 0 145 M NaCl was diluted twofold serially with 0 145 M NaCl To 0 4 ml portions was added 0 1 ml ion solution (conc 10 times final conc wanted) in 0 145 M NaCl and 0 5 ml 1 per cent human red cell suspension in 0 1 M tris buffer pH 7 6 containing 0 130 M NaCl The mixture was treated according to the test method described
Abscissa *Final conc of the cation*
Ordinate A mixture, to which was added 0 145 M NaCl *instead* of ion solution was employed as zero point (−100 per cent activation – complete inhibition)

TABLE 1 *Inactivation at Different Temperatures Expressed as Per Cent Remaining Haemolytic Activity*

Incubation temperature °C	Duration of incubation min						
	2	5	10	15	30	60	120
22					100	100	92
37				100	93	92	71
45	54	24	16	4	0		
52	41	0					
72	0						

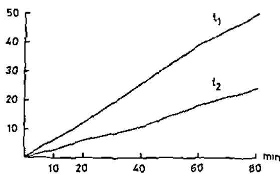


Fig 5 Haemolytic Activity Related to Incubation Time and Temperature
$t_1 = 37°$ C, $t_2 = 25°$ C
Temp coefficient

$$Q_{10°C} \; \frac{HU/ml_{t_1} \times 10}{HU/ml_{t_2} \times (t_1 \; t_2)} - 1\;8$$

Haemolysis was influenced by incubation time and temperature (Fig 5) At 37°S the curve was essentially rectilinear for 60 min

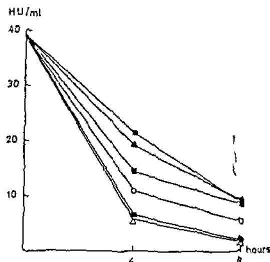
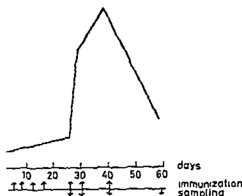


Fig 6 Effect of Proteases on Haemolytic Activity Trypsine α-chymotrypsine and pronase were dissolved in 0 04 M tris buffer pH 7 6 containing 0 145 M NaCl conc being 0 5 0 5 and 1 0 mg/ml respectively A papain solution of 0 5 mg/ml was made in 0 2 M phosphate buffer pH 7 4 containing 0 02 M cystein HCl and 0 004 M EDTA Equal volumes crude filtrate and enzyme solutions were incubated at 37°C frequently shaken and examined for remaining haemolytic activity after gentle cooling (to avoid precipitation of cystein) Blanks devoid of the respective proteases were included

Abscissa Duration of the incubation period
- ● papain blank
- ▲ pronase trypsine and α chymotrypsine blank
- ■ trypsine (enzyme/protein ratio 1 20)
- α chymotrypsine (enzyme/protein ratio 1 20)
- pronase enzyme/protein ratio 1 10)
- papain enzyme/protein ratio 1 20)

cells of types A AB, B and O showed haemolytic titres of 35 8, 35 7, 40 1 and 39 7 respectively The influence of pH on haemolysis is shown in Fig 3 A plateau was found between pH 6 8 and 7 6, which lies within the tolerable pH interval of red cells The tris buffer yielded higher titres than the phosphate buffer at pH 7 2 Activation and inhibition by ions are shown in Fig 4 Mg^{++} and Mn^{++} enhanced the haemolytic activity, while low concentrations of Zn and Hg had a strongly inhibitory action K had no effect in the concentrations examined, while Ca^{++} excerted a biphasic effect If Ca^{++} was added to undialysed filtrate a precipitate, obviously calcium phosphate was formed, the haemolytic activity disappearing simultaneously



Antihaemolytic Titres in Serum of Rabbit *inized with Haemolytic Crude Filtrate* mination of antihaemolytic activity was car out by twofold serial dilution of the sera in M NaCl, so that 0 25 ml remained in each Crude filtrate was diluted 1 in 4 in 0 03 M hate buffer pH 7 4, containing 0 080 M 0 25 ml being added to each tube After ation at 22°C for 2 hrs 0 5 ml 1 per cent red uspension was added and the tubes were according to the test method described antihaemolytic titre was calculated on the of 50 per cent inhibition of haemolysis the ocal value of the serum dilution being em \ as the titre
Intravenous injection of 0 5 ml crude filtrate Antiserum sampling

nean temperature coefficient for the in- 25–37°C ($Q_{10°C}$) was 1 8 No signific ifference in rate of haemolysis was seen oncentrations of 1 per cent and 2 per ed cell suspensions, while lowering the ntration to 0 5 per cent decreased the f haemolysis moderately

haemolytic material was not dialysable vation at different temperatures is in Table 1 A slow decrease in activity ed at 37°C, while at 45°C inactivation ed rapidly Inactivation by proteolytic es is shown in fig 6 After 4 hrs the was marked, being most pronounced in apain and pronase series After 8 hrs, ontaneous degradation of the haemolytic y made the specific effect less pro ed

Immunization of rabbits with crude filtrate (Fig 7) resulted in a marked increase in the antihaemolytic titre with maximum values after 40 days, followed by a decrease when the sampling and immunization interval was increased to 18 days

DISCUSSION

The haemolytic strains of Alcaligenes, described by Henriksen (6), reduced nitrates to nitrites and did not ferment glucose Apart from these reactions, the strains showed many similarities with the *A calcoaceticus* strain described The haemolytic properties were also similar, inasmuch as both were rapidly inactivated at 50°C It is possible, therefore, that the haemolysin here described is identical to the haemolysin of the strains described by Henriksen

The non dialysability and the sensitivity to proteases and heating indicate that the haemolytic effect is dependent upon a protein component

It is well established that many bacterial haemolysins possess enzymatic activities, *e g* the phopholipase activity of α haemolysin from *Clostridium perfringens* (7) The influence of pH, ions and incubation temperature makes it probable that the haemolytic activity of *A calcoaceticus* is due to an enzymatic action on the red cell membrane

The rapid inactivation of the haemolytic material on peptone free plates spread peripherically from the edge of the colonies and was thus probably due to other soluble bacterial products The protective effect of peptone might be explained by substrate competition, and could indicate that the haemolytic activity were attached to a protein or peptide component, vulnerable to the proteolytic activity of the mother organism Evidence of proteolytic activity was demonstrated by the lysis of a blood plasma coagulum, but has not been further investigated The presence of proteases might also explain the failure to produce haemolytic crude filtrates at 37°C and the spontaneous decrease in haemolytic activity of filtrate incubated at 37°C

It would be easier to separate and characterize the haemolytic material if it were possible to obtain a chemically defined medium, preferably of low molecular composition, which allowed production of high haemolytic titres

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PHAGOCYTIC ACTIVITY OF NEUTROPHILIC LEUKOCYTES OF A 2 INFLUENZA PATIENTS

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The ability of blood neutrophils of 18 serologically confirmed influenza patients to ingest killed *Staphylococcus epidermidis* was tested *in vitro*, and compared with 15 controls to determine whether defective phagocytosis might contribute to superimposed bacterial infec tions in influenza Phagocytosis was normal at the acute phase of disease, but two weeks later the activity was significantly increased, this was not due to serum factors and probably the cells themselves were unusually active Normal ingestion of bacteria by blood neutrophils at the acute phase does not suggest defective phagocytosis as a cause of bacterial com plications in influenza, but a local impairment in respiratory organs cannot be excluded

enza patients are abnormally susceptible cterial infections in respiratory organs fact well known in practice, has been med in clinical investigations (4, 17) ınimal experiments (4) Several factors been suggested as causes of this sus ıility Virus damages the mucous mem and impairs the ciliary function thus ıng the resistance of mucous membranes teria (4, 8, 9) Exudates form a favour nedium for bacterial growth (7) Leu ıa, especially neutropenia, is a common ıg in influenza, and the number of ophils that can be mobilized to infec- ıtes has been supposed to be abnormally) In several studies it has been shown influenza virus impairs the *in vitro* cytic ability of phagocytes from differ nimal species (5 10, 12, 15) *Wilson et al* (18) found disturbances in blood granulocyte phagocytosis in monkeys with influenza disease Thus impaired phagocyte function has been considered as one possible reason for the susceptibility to superimposed bacterial infections in influenza (15) During the influenza epidemic A 2 Hong Kong in winter 1969 we investigated the phagocytic activity of blood neutrophils from influenza patients

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MATERIAL AND METHODS

Patients and Controls

Material consisted of 18 young, otherwise healthy adult influenza patients, mainly students The diagnosis was confirmed serologically Four fold increase in HI titers from acute phase samples to samples taken two weeks later was considered diagnostic Healthy subjects, who had not had influenza or any other respiratory infections during this epidemic, and who had not been vaccinated against influenza were also studied as controls The age distribution of patients and controls was similar

Leukocytes

Venous blood was taken into a test tube containing heparin, 0 1 *mg/ml* blood (Heparin Medica, Medica Helsinki) Red cells were sedimentated by Boyum s method (2) Thirteen ml blood was layered in a 24 × 115 mm test tube on 10 ml solution containing methyl cellulose (Methocel 25 cP, Fluka AG, Switzerland, 2 per cent solution in distilled water), and sodium metrizoate (Isopaque, Medica 60 per cent, diluted with distilled water to 33 per cent solution) in proportion 16 10 Erythrocytes aggregated at the interface and fell to the bottom in about 40 min, leaving plasma with most of the leukocytes and platelets in the upmost layer The leukocyte plasma suspension was collected, and the neutrophil concentration was counted by conventional methods The suspension was centrifuged for 10 min at 125 g As much plasma was removed from the surface, so that the cell pellet suspended in the remaining plasma gave a neutrophil concentration of 5,6 × 10^6/ml Sometimes the cell suspension had to be diluted rather than concentrated, this was done with autologous plasma

Sterile glassware and siliconized tubes were used throughout

Bacteria

Staphylococcus epidermidis was grown for 20 hours in broth, and killed with formalin (0 05 ml/10 ml broth) The bacteria were washed three times in saline, and finally suspended in saline to an optical density known to correspond to 3 × 10^9 bacteria/ml This suspension was divided into 1 ml portions and kept at —40° C For each experiment one portion of bacterial suspension was thawed and diluted 1 2 with saline This was the final concentration of bacterial suspension Preliminary experiments had shown that deep frozen bacterial suspension keeps for several months

Leukocyte—Bacteria Suspensions and Quantitation of Phagocytosis

A volume of 1 35 ml of leukocyte suspension was pipetted into 10 × 98 mm test tubes and kept for 10 min at 37° C 0 15 ml of bacterial suspension was then added giving the final neutrophil concentration of 5 × 10^6/ml and the ratio of bacteria to neutrophils 3 1 Tubes were closed with sterile rubber stoppers, mixed by turning 20 times end over end and left to stand at 37° C After 6 min the tubes were mixed again as above

TABLE 1 *Percentage Phagocytosis (%P) and Phagocytic Index (PI) Values of Patient and Control Groups after 6 and 20 min Incubation at the Acute Phase 2 Weeks Later and 4 Months Later*

	Number of subjects	6 min incubation		20 min incubation	
		%P	PI	%P	PI
Acute phase					
patients	18	45 ± 9	0 71 ± 0 21	70 ± 6	1 42 ± 0 25
controls	17	45 ± 7	0 72 ± 0 17	68 ± 8	1 47 ± 0 26
2 weeks later					
patients	18	53 ± 7*	0 87 ± 0 20	75 ± 5*	1 66 ± 0 22
controls	6	45 ± 7	0 77 ± 0 21	66 ± 10	1 41 ± 0 31
4 months later					
patients	15	45 ± 7	0 69 ± 0 19	69 ± 6	1 43 ± 0 24
controls	10	42 ± 10	0 66 ± 0 19	71 ± 6	1 45 ± 0 23
Significance of changes in patient group (p)					
Increase acute phase—2 weeks		< 0 01	< 0 05	< 0 05	< 0 05
Decrease 2 weeks–4 months		< 0 01	< 0 05	< 0 05	NS

Arithmetic means ± S D are given

* Difference from control significant ($p<0.05$)

NS = not significant

TABLE 2 *Changes in Results of Individual Patients between Different Sampling Times*

	6 min incubation		20 min incubation	
	%P	PI	%P	PI
nge between				
e phase and 2 weeks				
Increased	15	14	11	15
Unchanged	–	–	2	–
Decreased	3	4	5	3
p	– 0 01	– 0 05	NS	= 0 01
nge between				
eks and 4 months				
Increased	2	3	2	2
Decreased	13	12	13	13
p	– 0 01	– 0 05	– 0 01	= 0 01

gures show the numbers of patients whose results behaved as indicated in the table
values ind cate the significance of rising or falling tendency
or further explanation see Table 1

samples were taken for smears The same edure was repeated after 20 min incubation smears were stained with Wright s stain entage phagocytosis (percentage of neutrophils had ingested bacteria) and phagocyt c index rage number of bacteria in one neutrophil) determined for 100 neutrophils

istical Methods

atistical calculations were based on binomial ibution and t test.

RESULTS

itrophils of patients were tested at the te phase of disease usually on the second hird day after the onset of fever At this all patients had fever and other in nzalike symptoms In the same series of eriments neutrophils from 17 control sub s were also examined Two weeks later samples were taken to discover the nor values of patients after convalescence patients had recovered without compli ons and in all cases fever had disappeared in a week At this time all were well pt for a dry cough in some, and promi t fatigue in one Since unexpected results obtained at this phase one further set amples was collected from the patients r months after the acute phase Fifteen of original 18 patients were contacted and examined at that time Further controls, six at two weeks and ten at four months were studied

Table 1 shows the results from patients and controls after 6 and 20 min incubation at the acute phase, two weeks and four months later At the acute phase no differ ences between patients and controls can be seen, but at two weeks the cells of influenza patients seem to be more actively phagocytic than controls The percentage differences are significant those of indices are not In four months samples there are again no differ ences The increase in the results of patients from acute phase to two weeks phase, and decrease from two weeks to four months, are statistically significant events, as shown in Table 1

The changes in results of individual pa tients from phase to phase are followed in Table 2 The 6 min percentage phagocytosis is higher two weeks after the acute phase than at the acute phase in 15 of the 18 pa tients and the same tendency can be ob served in other parameters The trend to enhanced phatocytosis at two weeks is signi ficant at p levels indicated in Table 2 The opposite trend is seen between two weeks and four months The decrease is significant in all parameters A corresponding compari son cannot be presented for the controls be

TABLE 3 *Phagocytosis by Normal Neutrophils in Sera from Patients and Controls*

Incubation medium	6 min incubation		20 min incubation	
	%P	PI	%P	PI
Acute phase sera	64 ± 6	1 20 ± 0 15	86 ± 6	2 19 ± 0 25
2 weeks phase sera	63 ± 5	1 19 ± 0 17	80 ± 4	2 07 ± 0 24
Control sera	64 ± 4	1 24 ± 0 19	81 ± 6	2 03 ± 0 29

Each group of sera consists of 10 samples
For further explanation see Table 1

cause these were different persons at different times However, in the controls as a whole there were no differences at different *times of sampling* compared with the patient group increase at two weeks

The increased activity of patient cells in two weeks samples might be either due to some phagocytosis promoting serum factor, or the cells themselves may be better phagocytes To study this, neutrophils from healthy subjects were allowed to ingest bacteria in sera collected from patients at the acute phase, and 2 weeks later, and in sera from controls studied simultaneously All sera had been kept in —40° C Each group consisted of ten sera Sera from acute phase and two weeks phase were *from the same patients Cells of each of the four donors* were *allowed* to ingest bacteria in equal numbers of sera from each group Cells and sera were from persons of same ABO blood group in every experiment No differences between serum groups could be detected, as seen in Table 3 This speaks against the existence of phagocytosis promoting serum factors and indicates that the phagocytes themselves must be unusually active The slightly higher levels of results *in these experiments compared* with others, is probably due to the fact that serum was used as medium instead of plasma

Since staphylococci were used as particles for ingestion, antistaphylolysin titres of patients were examined at the acute phase and two weeks later The titres remained unchanged

DISCUSSION

In the light of results obtained in these experiments it would seem that the phagocytic activity of blood neutrophils of influenza patients is normal at the acute phase of the disease, and no disturbances can then be seen This does not, however, rule out the possibility of local defects in phagocytosis in the respiratory organs themselves Influenza is mainly a disease of the respiratory tract, and abnormal susceptibility to bacterial infections has not been described elsewhere in the body It is possible that leukocytes coming into direct contact with virus in the lungs and respiratory pathways are damaged and ingest bacteria ineffectively, without there being *any evidence of such damage in circulating blood neutrophils* On the other hand, many systemic effects appear in influenza such as leukopenia, unusually prominent head and muscle ache, and a strong effect on general condition Viremia occurs, although probably not very often (13, 16), and in fatal cases virus has been isolated from several organs (1, 14) These facts indicate that many functions outside of respiratory organs may also be disturbed Deficient phagocytic *ability of blood granulocytes has been shown* in monkeys with influenza disease (18) It is also of interest that impaired phagocytic function of peripheral blood leukocytes has been demonstrated by *Kantoch et al* (11) in patients with another viral disease, virus hepatitis This impairment was probably due to the presence of virus itself in leukocytes since sera of patients did not affect the activity of leukocytes from healthy subjects

wo weeks after the acute phase the ocytic activity of leukocytes from in- iza patients was increased The cause of phenomenon is not clear If it were due ome phagocytosis promoting serum fac- one would have expected that normal would have become more phagocytic n incubated in sera from two weeks , than in sera from acute phase, or in rol sera No differences between differ- sera were, however, observed This indi- s indirectly that the cells themselves are active phagocytes Direct evidence for i e enhanced activity of patient cells iormal serum as compared with control under the same conditions, could not be ined due to unfortunate practical diffi ies It seems probable, however, that the inced phagocytosis is a property of the themselves Neutropenia often occurs nfluenza, how this is produced, is not wn It has been proposed that influenza resses the function of bone marrow, and this would cause diminished production eutrophils However in influenza a "shift eft" in blood picture can often be seen

We afterwards studied the records of ienza patients in Aurora Municipal Hos l Earliest blood pictures had been taken he fourth day of disease In several cases found clearly elevated percentages of d form neutrophils Leukocyte responses uperimposed bacterial infections in influ- i is good (6) These facts speak against a irbance in bone marrow function A shift the left indicates that the relative pro- tion of young neutrophils is increased In convalescent phase when normal neutro- counts are returning after the neutro- ic phase, the relative proportion of young trophils may be elevated This may be a on for the increased phagocytic activity he convalescent phase, since young neu phils have been shown to be better phago s than older cells (3)

he method used measures phagocytosis narrow sense, i e the engulfment phase, stion This has also been measured in all vious work concerning interactions of in fluenza virus and leukocytes *in vitro* or *in vivo* Undisturbed ingestion does not, however, give any information concerning the bactericidal properties of leukocytes, of which chronic granulomatous disease of childhood is a good example The effect of influenza virus on the intracellular fate of bacteria remains to be investigated

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This investigation was supported by the *Sigrid Jusélius Foundation* and by the *National Research Council for Medical Sciences*, Helsinki, Finland

Timo Palosuo, M D, Department of Virology, University of Helsinki, kindly made the influenza antibody determinations

We thank prof *Erkki Klemola* for allowing us to study records of patients in Aurora Municipal Hospital

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AN IMMUNODIFFUSION MICROMETHOD FOR COMPARATIVE ANALYSIS OF THE HEMOLYTIC ACTIVITY AND ANTIGENICITY OF STREPTOLYSIN O

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An immunodiffusion micromethod for determination and characterization of hemolytic activity based on streptolysin O experiments is described The hemolytic activity of Sl O and its inhibition by its corresponding antibody is illustrated The technique offers an alternative way to identify hemolytically active antigens among other precipitating factors in a crude antigen preparation

mmunological studies of enzymes the lation between antigenicity and biologic ity offers an intriguing problem (*Uriel vrameas* 1964) Mixing of an enzyme its corresponding antibody *in vitro* is lly accompanied with an inhibition of enzymatic activity although exceptions known (*Cinader* 1963 *Cinader* 1967, *tra* & *Arnon* 1968) In immunological ses employing diffusion in gel methods nzymatic activity is usually unchanged ven more pronounced in the enzyme enzyme precipitate The characterization e enzyme complexed with its correspond-ntibody might therefore be performed ective of the presence of other immune pitates in the antigen-antibody mixture *l* 1966) This is of special value in ana-of biologically active antigenic prepara-which though advanced techniques have been employed in the purification procedure, still shows the presence of contaminants reacting with their corresponding antibodies Extracellular material from group A streptococci may serve as a good example as it may contain more than 20 different antigens of toxic and enzymatic nature (*McCarty* 1965) Many attempts have also been made to purify these antigens and some of the streptococcal enzymes have been at least partially purified (*Halbert* & *Auerbach* 1961 a) One of these, which have been most intensively studied—streptolysin O—has never been purified though extensive attempts have been made (*Halbert* & *Auerbach* 1961 a, *Alouf et al* 1965) This is of special interest since streptolysin O has been shown to possess cardiotoxic properties (*Halbert et al* 1961 b, *Reitz et al* 1968)

The aim of the present investigation was to find a simple method to identify streptolysin O activity and to correlate this to a

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particular immunoprecipitate in a multiple streptococcal antigen antibody system

MATERIALS AND METHODS

Antigenic Preparations

Streptococcal extracellular material (EC) The streptococcal strain S84 (Lancefield group A, Griffith type 3) was cultivated in the antigen free trypticase yeast autolysate medium under stabilized pH conditions at pH 6 8 for 16 hours as described by *Holm & Falsen* (1967) After centrifugation at 10000 × g for 30 minutes the supernatant was precipitated by dialysis against 10 vol of 3 76 M $(NH_4)_2SO_4$ at 4° C for 24 h The precipitate was dissolved in 1/20 vol of buffered saline (pH 6 8) and finally concentrated to 1/100 vol by dialysis against 30 per cent polyethylene glucol 3000

Streptococcal intracellular material (IC) The streptococcal cells from the above mentioned culture were ultrasonicated and freeze pressed as described by *Holm* (1967)

Streptolysin O (Sl O) determination The hemolytic activity of Sl O was determined as described by *Alouf et al* (1965) and expressed in HD_{50} The combining capacity of Sl O (HU) with its corresponding antibody was also determined according to the method of *Alouf et al* (1965) The streptococcal culture after centrifugation had a Sl O activity of 25 HU/ml which corresponded to 1000 HD_{50}/ml

Anti streptolysin O (ASO) determination ASO determinations were performed according to the technique of *Kalbak* (1947)

Antisera

Sheep antistreptococcal serum (anti IC + EC) was obtained from a sheep which had been hyper immunized by repeated injections of streptococcal IC and EC preparations as described by *Holm* (1967) The ASO titer of this serum was 800 ASO units/ml

Human antistreptococcal serum (H ASO) A pool of sera from 50 patients with recent streptococcal infections each of them showing an ASO titer of 800 units/ml or more was precipitated with $(NH_4)_2SO_4$ following the procedure for gamma globulin precipitation used by *Kendall* (1937) The final preparation was about 8 times concentrated as judged by its ASO titer (8000 ASO units/ml)

Diffusion in gel analyses The immunodiffusion method of *Ouchterlony* (1949) was employed in a gel chamber micromodification described by *Wadsworth* 1962 but without addition of pre

lution of 2 per cent agar (Nobel Agar, Difco) in buffered saline (pH 6 8) was brought to 50° C and mixed with an equal volume of a 5 times washed warmed suspension of sheep erythrocytes (2 per cent) This blood agar mixture replaced the 1 per cent saline agar usually used in the micro plate technique In all other respects the micro chamber technique of *Wadsworth* (1962) was employed

Cholesterol suspension 0 1 g cholesterol (Fisher Scientific Company) was dissolved in 10 ml 96 per cent ethanol 1 ml of this solution was transferred to 100 ml saline and shaken vigorously at 23° C for 20 minutes and allowed to sediment This supernate was used in streptolysin O inhibition experiments

Lecithin suspension A lecithin suspension (Lecithinum ex ovo puriss, E Merck AG, Darmstadt) was prepared as described for the cholesterol suspension

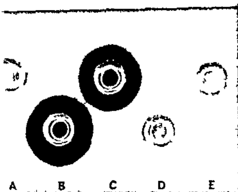
Streptolysin O reduction The streptococcal EC material was reduced by adding 0 04 ml of a 10 per cent solution of L(±) cysteine hydrochloride (AB Kebo Stockholm) to each ml of EC material After 20 minutes at room temperature the material was considered fully reduced

Oxidation The streptococcal EC material was oxidized by addition of an equal amount of 3 per cent H_2O_2 which was prepared from a 30 per cent solution by dilution in saline The mixture was shaken vigorously for 1 hour at room temperature in a big bottle holding 100 times the volume of the mixture

EXPERIMENTS AND RESULTS

Characterization of the Hemolysis Induced by Streptococcal EC Material in Blood Agar Micro Chamber

To study the characteristics of the hemolysis induced by streptococcal extracellular material a batch of EC material was reduced by cysteine hydrochloride After incubation at room temperature for 20 minutes the material was divided into 5 portions To the first portion was added an equal volume of cholesterol suspension and to portion two the same amount of lecithin The third portion was oxidized by H_2O_2 while to the fourth portion was added an equal volume of H-ASO The four portions and a fifth one, to which was only added an equal amount of saline, were incubated at room temperature for 2 hours and 0 02 ml of each filled into the wells of the blood agar micro chamber



1 Photo of a blood agar microplate showing clear hemolytic area around the well de ted (B) containing reduced EC and the well containing reduced EC + lecithin The blood id the other wells—containing oxidized (A), sterol treated (D) and H ASO treated EC (E) s unaffected

he experiment was performed at room perature (about 23° C) in a moist cham and the lysis was followed by repeated rvations and photographic registrations 1 represents a photo of the reactions 4 hours of diffusion As may be seen vell (B) containing reduced EC material the centre well (C) containing reduced material incubated with lecithin were surrounded by a clear zone A corre ding zone was not detectable with the rial filled in the other wells The lytic s grew during the first 12 hours but incu n of the plates for longer times did not antially increase the hemolytic zones In same experiment it was noted that at stages a small hemolytic zone started to lop around the well containing the oxi l material This was probably caused by tion of the oxidized material by diffu of reducing substances from the sur ding wells and a simultaneous decrease e oxidizing capacity of the H_2O_2 added us material The plates were kept at temperature for at least two days with obvious distortion of the hemolytic

Inhibition of the Hemolytic Zone by H-ASO

The suitability of using hemolysis as an indicator in determination of the position of a hemolytic antihemolytic precipitation im mune system was studied in the following way (see Fig 2) The centre well was filled with reduced streptococcal EC material and the left and the right well with H ASO and anti IC + EC respectively The reactions were followed by daily photographic regis trations The first picture (Fig 2 A) was taken 4 hours after the start The hemolytic area was almost circular but a slight sign of flattening may be noticed at position 10 o clock Three hours later (Fig 2 B) a clear cut zone of inhibition could be seen at the same position and, although it may not be detectable on the photo, a precipitation band started to build up Also at position 2 o'clock a flattening of the circle could be noted Five hours later (Fig 2 C) two precipitation bands could be seen at position 10 o'clock and furthermore one at position 2 o'clock Two days later the plexi glass template was lifted off, and another photo was taken (Fig 2 D) The agar plate was placed in a Petri dish filled with 10 ml saline To the saline was added 1 ml staphylolysin (30 IE) and the plate was incubated at 37° C for 2 hours After rinsing with saline another photo was taken (Fig 2 E) As may be seen the erythrocytes in the earlier unaffected parts of the blood agar layer was now completely lysed As a result of this the faint precipitation bands could now be seen which had earlier been hidden by the presence of not hemo lyzed erythrocytes By comparison between the zone of inhibition at position 10 and 2 o'clock in Figs 2 C and 2 D and the precipi tates seen on the final photo in Fig 2 E it was possible to correlate the zones of inhibi tion to a certain precipitation band Further more the lysis inhibiting precipitation band at either side coalesced Only one of the precipitates could be related to the zone of inhibition of the hemolysis although precipi tates could be found on either side of this zone

particular immunoprecipitate in a multiple streptococcal antigen antibody system

MATERIALS AND METHODS

Antigenic Preparations

Streptococcal extracellular material (EC) The streptococcal strain S84 (Lancefield group A, Griffith type 3) was cultivated in the antigen free trypticase yeast autolysate medium under stabilized pH conditions at pH 6 8 for 16 hours as described by *Holm & Falsen* (1967) After centrifugation at 10000 × g for 30 minutes the supernatant was precipitated by dialysis against 10 vol of 3 76 M $(NH_4)_2SO_4$ at 4° C for 24 h The precipitate was dissolved in 1/20 vol of buffered saline (pH 6 8) and finally concentrated to 1/100 vol by dialysis against 30 per cent polyethylene glucol 3000

Streptococcal intracellular material (IC) The streptococcal cells from the above mentioned culture were ultrasonicated and freeze pressed as described by *Holm* (1967)

Streptolysin O (SlO) determination The hemolytic activity of SlO was determined as described by *Alouf et al* (1965) and expressed in HD_{50} The combining capacity of SlO (HU) with its corresponding antibody was also determined according to the method of *Alouf et al* (1965) The streptococcal culture after centrifugation had a SlO activity of 25 HU/ml which corresponded to 1000 HD_{50}/ml

Anti streptolysin O (ASO) determination ASO determinations were performed according to the technique of *Kalbak* (1947)

Antisera

Sheep antistreptococcal serum (anti IC + EC) was obtained from a sheep which had been hyperimmunized by repeated injections of streptococcal IC and EC preparations as described by *Holm* (1967) The ASO titer of this serum was 800 ASO units/ml

Human antistreptococcal serum (H ASO) A pool of sera from 50 patients with recent streptococcal infections each of them showing an ASO titer of 800 units/ml or more was precipitated with $(NH_4)_2SO_4$ following the procedure for gamma globulin precipitation used by *Kendall* (1937) The final preparation was about 8 times concentrated as judged by its ASO titer (8000 ASO units/ml)

Diffusion in gel analyses The immunodiffusion method of *Ouchterlony* (1949) was employed in a gel chamber micromodification described by *Wadsworth* 1962 but without addition of preservatives Registrations of the reactions were made by Polaroid panchromatic film reproductions

Immunodiffusion in blood agar chamber A solution of 2 per cent agar (Nobel Agar, Difco) in buffered saline (pH 6 8) was brought to 50° C and mixed with an equal volume of a 5 times washed warmed suspension of sheep erythrocytes (2 per cent) This blood agar mixture replaced the 1 per cent saline agar usually used in the micro plate technique In all other respects the micro chamber technique of *Wadsworth* (1962) was employed

Cholesterol suspension 0 1 g cholesterol (Fisher Scientific Company) was dissolved in 10 ml 96 per cent ethanol 1 ml of this solution was transferred to 100 ml saline and shaken vigorously at 23° C for 20 minutes and allowed to sediment This supernate was used in streptolysin O inhibition experiments

Lecithin suspension A lecithin suspension (Lecithinum ex ovo puriss, F Merck AG Darmstadt) was prepared as described for the cholesterol suspension

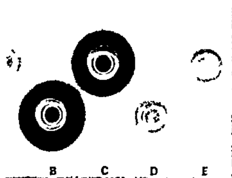
Streptolysin O reduction The streptococcal EC material was reduced by adding 0 04 ml of a 10 per cent solution of L(±) cysteine hydrochloride (AB Kebo, Stockholm) to each ml of EC material After 20 minutes at room temperature the material was considered fully reduced

Oxidation The streptococcal EC material was oxidized by addition of an equal amount of 3 per cent H_2O_2, which was prepared from a 30 per cent solution by dilution in saline The mixture was shaken vigorously for 1 hour at room temperature in a big bottle holding 100 times the volume of the mixture

EXPERIMENTS AND RESULTS

Characterization of the Hemolysis Induced by Streptococcal EC Material in Blood Agar Micro Chamber

To study the characteristics of the hemolysis induced by streptococcal extracellular material a batch of EC material was reduced by cysteine hydrochloride After incubation at room temperature for 20 minutes the material was divided into 5 portions To the first portion was added an equal volume of cholesterol suspension and to portion two the same amount of lecithin The third portion was oxidized by H_2O_2 while to the fourth portion was added an equal volume of H-ASO The four portions and a fifth one, to which was only added an equal amount of saline, were incubated at room temperature for 2 hours and 0 02 ml of each filled into the wells of the blood agar micro chamber

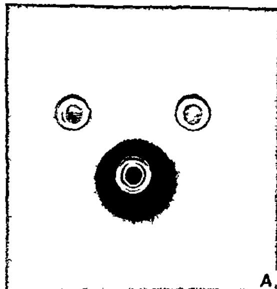


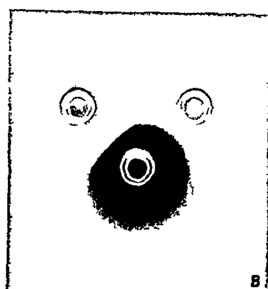
Pl oto of a blood agar m croplate sl owing lear hemolytic area around the well de (B) containing reduced EC and the well ontaining reduced EC + lecithin The blood the other wells—containing oxid zed (A), terol treated (D) and H ASO treated EC (E) unaffected

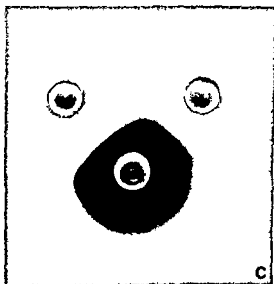
experiment was performed at room ture (about 23° C) in a moist cham the lysis was followed by repeated ations and photographic registrations 1 represents a photo of the reactions 4 hours of diffusion As may be seen ell (B) containing reduced EC material e centre well (C) containing reduced a incubated with lecithin were by a clear zone A corre ng zone was not detectable with the l filled in the other wells The lytic grew during the first 12 hours but incu of the plates for longer times did not ntially increase the hemolytic zones In experiment it was noted that at ages a small hemolytic zone started to around the well containing the oxi al This was probably caused by n of the oxidized material by diffu f reducing substances from the sur ng wells and a simultaneous decrease oxidizing capacity of the H_2O_2 added s material The plates were kept at erature for at least two days with vious distortion of the hemolytic

Inhibition of the Hemolytic Zone by H ASO

The suitability of using hemolysis as an indicator in determination of the position of a hemolytic antihemolytic precipitation im mune system was studied in the following way (see Fig 2) The centre well was filled with reduced streptococcal EC material and the left and the right well with H ASO and anti IC + EC respectively The reactions were followed by daily photographic regis trations The first picture (Fig 2 A) was taken 4 hours after the start The hemolytic area was almost circular but a slight sign of flattening may be noticed at position 10 o clock Three hours later (Fig 2 B) a clear cut zone of inhibition could be seen at the same position, and, although it may not be detectable on the photo, a precipitation band started to build up Also at position 2 o clock a flattening of the circle could be noted Five hours later (Fig 2 C) two precipitation bands could be seen at position 10 o'clock and furthermore one at position 2 o clock Two days later the plexi glass template was lifted off, and another photo was taken (Fig 2 D) The agar plate was placed in a Petri dish filled with 10 ml saline To the saline was added 1 ml staphylolysin (30 IE) and the plate was incubated at 37° C for 2 hours After rinsing with saline another photo was taken (Fig 2 E) As may be seen the erythrocytes in the earlier unaffected parts of the blood agar layer was now completely lysed As a result of this, the faint precipitation bands could now be seen which had earlier been hidden by the presence of not hemolyzed erythrocytes By comparison betwee the zone of inhibition at position 10 and o clock in Figs 2 C and 2 D and the precip tates seen on the final photo in Fig 2 E was possible to correlate the zones of inhib tion to a certain precipitation band Further more the lysis inhibiting precipitation ban at either side coalesced Only one of th precipitates could be related to the zone o inhibition of the hemolysis although precip tates could be found on either side of th zone









D



E

Fig 2 Photographic recording illustrating the development of hemolysis zones of inhibition and precipitates The photos were taken 4 hours (A) 7 hours (B) 12 hours (C) and 3 days (D and E) after the start of the experiment The lower well contained reduced FC material while the left well was filled with H ASO and the right one with anti IC + EC Photo E was taken after hemolysis of earlier unchanged erythrocytes

DISCUSSION

ral experimental methods have been de-
ʞed in order to correlate a hemolytic ac-
y to an antigen Thus *Hanson & Holm* (1) were able to localize the hemolytic vity of streptolysin O after agar gel electrophoresis of a crude extracellular strepto al material *Mansa & Kjems* (1965) rmined the mobility of antibodies to alfa hylolysin by an immunoelectrophoretic iolysis inhibiting technique These au-s (1968) also applied the same technique tudies on antistreptolysin O activity with-M components of humansera The fuz ss of the zones of inhibition has limited value of these methods and has probably n the main cause of disagreement on *e g* electrophoretic mobility of streptolysin O *ock & Uriel* 1961)

he characterization of streptolysin O as antigenic factor capable of hemolyzing a e variety of mammalian, avian and am nan erythrocytes in its reduced form is nly attributed to *Herbert & Todd* (1941) *ntt & Todd* (1939) also found that min amounts of cholesterol but not lecithin tivated Sl O

n the present investigation the suitability blood agar micro chamber technique for ction of streptolysin O was tested on the s of these characteristics The lytic ac y of reduced streptococcal EC material heep erythrocytes also in the presence of thin was easily recognized already after hours of diffusion and a complete in tion in the presence of specific Sl O antiies and cholesterol could be registered as l as the lack of hemolytic activity of oxidi Sl O It therefore seems reasonable to ume that the hemolytic reactions noted responded to the presence of reduced)

n the second set of experiments attempts e made to correlate the Sl O ASO reac to an immune precipitate assuming that unneutralized Sl O could pass the im iospecific barrier that is the precipita band consisting of Sl O and its corresponding antibody The results show that an inhibition of the hemolysis could be noted already after four hours of diffusion At that time no precipitation band was detectable However, the immunospecific barrier had apparently already started to build up, since the further development of the reactions made it possible to establish a positive corre lation between inhibition of hemolysis and a specific immunoprecipitate The choice of two sera with different antibody composition —one from a gammaglobulin pool of sera with high ASO titers obtained from patients with recent streptococcal infections, the other from a sheep hyperimmunized with strepto coccal material but with a medium high ASO titer—enabled to role out sources of errors like unspecific inhibition by antigen antibody precipitates not correlated to Sl O ASO Furthermore, the position of the zone of inhibition seemed to correlate with the ASO activity of the two sera employed being closer to the well containing the sheep serum with the lower ASO titer Additional aspects on the quantitation of ASO and Sl O employing the present technique are under way

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This study was supported by a grant from *Statens Medicinska Forskningsråd* Sweden

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THE FORMATION OF STREPTOLYSIN O UNDER STABILIZED pH CONDITIONS

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Employing a system for continued pH adjustment and a growth medium containing factors essential for streptococcal growth and Sl O production the formation of Sl O at different growth phases was studied The Sl O formed during the early phase of exponential growth could not be completely neutralized by its corresponding antibody thus indicating a lack of or a lower antigenicity of this streptolysin The mechanism for Sl O formation is probably an active process where a feed back system might be involved

determination of antistreptolysin O anti ies (ASO) in patients' sera is still the t important serologic reaction to reveal a nt infection with beta hemolytic strepto i In laboratories performing this test the duction of streptolysin O (Sl O) of acceptable activity and storability is therefore urrent problem Although the factors es ial for the cultivation of beta hemolytic ptococci and the production of strepto i O have been studied by many authors *cIlwain* 1946 *Bernheimer & Pappenner* 1942 *Slade & Knox* 1950, *Zampieri l* 1960 *Slade et al* 1951, *Fox* 1961 and *kelson* 1964) most of the growth media imonly employed for Sl O production *e g* Todd Hewitt broth *(Todd & Hewitt 2)* are lacking one or the other of these wth factors The growth promoting capa is therefore often poor and the strepto i O produced usually loses its activity within a few months when such media are employed A growth medium composed according to available data in the literature on growth factors essential for streptococcal growth and Sl O formation was worked out by *Holm & Falsen* (1967) This medium seemed well suited for studies concerning the formation of extracellular streptococcal factors during the cultivation

The aim of the present investigation was to determine optimal pH for Sl O production to study the formation of Sl O at different times during the cultivation and to analyze the antigenicity of the Sl O preparations produced

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MATERIALS AND METHODS

Streptococcal strain Strain S84 Griffith s type 3 Lancefield s group A was employed throughout the whole investigation

Growth medium All cultivations were performed in the trypticase yeast autolysate base medium with

supplements and glucose (1 per cent) as described by *Holm & Falsen* (1967)

Cultivation method Batch cultivations were performed in Quickfit (Quickfit & Quartz Ltd, Staffordshire, England) wide neck 1 or 5 liters culture vessels employing the Quickfit stirring device During the cultivation period the pH was continously adjusted by means of a Metrohm impulsomat (E 373) and Titrigraph (Metrohm Ltd, Herisau, Switzerland) delivering 5N NaOH The consumption of NaOH was continously recorded The culture, which was inoculated with 1/30 volume of an 8 hours' culture of the S84 strain, was grown at 37° C and samples were withdrawn at intervals for determination of Sl O activity, glucose concentration and estimation of bacterial growth

Concentration of extracellular streptococcal antigens (EC) Samples taken from the culture at different intervals were sterilized by passing through membrane filters (Gottingen No 6), and precipitated by dialysis for 24 hours against 10 vol of saturated $(NH_4)_2SO_4$ (3 76 M) The precipitate was collected by centrifugation at 10,000 × g, dissolved in 1/20 volume of phosphate buffered saline and finally concentrated to 1/100 of the original volume by dialysis versus 30 per cent polyethylene glucol The concentrated antigens were designated EC followed by a number representing the time at the sampling (*e g* EC_6 = conrentrated antigen of a sample taken after 6 hours' cultivation)

Intracellular streptococcal antigen (IC) Five times washed streptococci—from a 16 hours' culture—were sonicated and freeze pressed as described before (*Holm & Kaijser* 1967) The supernatant after centrifugation at 10,000 × g was designated IC material

Estimation of bacterial growth Bacterial growth was estimated by determination of the optical density (O D) in a Beckman C apparatus using a red filter As the O D values gives a straight line relationship only within a limited range (0 4–0 6) the cultures of samples were always diluted to fall within these ranges All higher O D values given therefore represent multiplications of values registered after dilution of the culture in uninoculated medium According to the McFarland scale an O D of 0 40 approximates 6 10^8 streptococci/ml

Glucose determination The determination of glucose in the samples—after sterilization by filtration through Gottingen No 6 membrane filters—were performed by the enzymatic method employing Glox (Kabi, Stockholm)

Streptolysin O determinations Streptolysin O activity was registered in two different ways

a) *Hemolytic unit* (H U) Determination of the highest dilution of lysin giving a complete hemolysis of 0 5 ml of a 5 per cent suspension of sheep erythrocytes The reciprocal value of this dilution was expressed as the number of H U/ml (hemolytic unit)

b) *Todd units* 1 T U = the reciprocal of the lowest dilution of lysin which could be neutralized by 1 antistreptolysin unit using a standard antistreptolysin from Statens Seruminstitut, Copenhagen (*Kalbak* 1947) Todd found (1932) that 2 ½ times the hemolytic unit as described above corresponded to 1 T U

Streptococcal proteinase activity was measured by the milk clotting method described by *Elliott* (1945)

H ASO A concentrated gammaglobulin pool of sera from patients with a recent streptococcal infection and showing increased ASO titers was employed The ASO titers of this concentrated pool was 8 000 ASO units/ml

Anti IC + EC serum was collected from a sheep which had been hyperimmunized with extracellular and cellular material from group A streptococci (*Holm & Kaijser* 1967) The ASO titer of this serum was 800 units/ml

Diffusion in gel analyses Immunodiffusion was performed according to the technique of *Ouchterlony* (1949) in a micromodification as described by *Wadsworth* (1962) The newly described blood agar micro chamber technique for detection of hemolytic activity as well as inhibition of hemolysis correlated to the Sl O ASO immunoprecipitate was also employed (*Holm & Moller* 1971)

EXPERIMENTS AND RESULTS

Influence of pH upon Streptococcal Growth and Streptolysin O Formation

One batch of growth medium was divided into eight 1 liter portions and the pH adjusted to 5 5, 5 8, 6 2, 6 8, 7 0, 7 2 7 4 and 7 8 respectively Each batch was inoculated with an equal amount of an 8 hours old streptococcal start culture (O D 0 5-0 7) The original pH of each culture was maintained constant during a 16 hours cultivation period at 37° C

No growth was registered in the media adjusted to pH 5 5 or 5 8 However if after the incubation period the pH was raised to 6 2 the culture started to grow during the following 16 hours period at the same rate as the pH 6 2 culture The results concerning streptococcal growth, streptolysin O hemolytic activity and consumption of 5N NaOH after 16 hours incubation are listed in Ta

ABLE 1 *The Relationship between Bacterial Growth, NaOH Consumption and Sl O Activity at Different pH*

pH	Bacterial growth (in O D)	Consumption of 5N NaOH ml/l culture	Sl O activity H U./ml	Number of cultures
5 5	–	–	–	2
5 8	–	–	–	2
6 2	0 83–*1 0** 1 2	13–*16**–19	35–*55**–100	8
6 8	0 96–*1 3* –1 5	17 *21* –22	35 *50* –100	10
7 0	0 86–*1 2* –1 35	15–*19* –22	25–*45* –110	4
7 2	0 61–*0 74*–0 81	8 *9* –12	20–*24* – 30	3
7 4	0 54–*0 66*–0 78	6– *8* –10	<20	2
7 8	0 11–*0 16*–0 22	2– *3* – 4	< 5	2

The figures underlined are average values

1 As may be seen only slight differences e noted concerning these parameters from cultures at pH 6 2, 6 8 and 7 0, but al 'y a change of 0 2 pH units up to 7 2 *ilted* in a decrease in growth and con iption of NaOH which was accompanied ι a lower Sl O activity Above pH 7 4 a poor growth was obtained and the ac y of the cultures as measured by con ption of NaOH and Sl O release was low It may be mentioned that 100 H U esponds to 40 T U (Todd Units = bining units) In filtrates from a few of ~ cultures streptococcal proteinase ac y was estimated No proteinase activity found in any of the cultures grown at 6 8 or above but in four out of five of pH 6 2 cultures proteinase activity could lemonstrated and milk clotting was ach d already within 1 hour employing cul- filtrates diluted 1 10 The milk clotting d be completely inhibited by addition of acetate (10 ³ M)

luction of Sl O During Different Phases of Growth

wo streptococcal cultures, one grown at 6 2 and the other at pH 6 8 were studied sampling at short intervals As before, activity, consumption of 5N NaOH and O D were registered Fig 1 illustrates results of the pH 6 8 culture concerning NaOH consumption and O D Similar results were obtained with the pH 6 2 culture It may be noted that the logarithmic growth phase stopped at a time when only half the amount of the final consumption of NaOH was reached Sl O activity was not detected during the first four hours of cultivation but a slight activity was registered after 8 hours in the pH 6 8 cultures (5 H U /ml) and reached its maximum in the sample taken after 12 hours' of growth (50 H U /ml) No decline in activity was noted during the following 12 hours of cultivation In this respect the pH 6 2 culture behaved differently Sl O activity was not found during the first four hours Maximal activity was registered in the sample taken after 8 hours' of cultivation (50 H U /ml) The same activity was demonstrated up to 16 hours' of cultivation after which time it gradually decreased to 15 H U that was found in the 24 hours' sample It may be noted that in these experiments Sl O activity could only be demonstrated in samples taken 4–8 hours after the end of the logarithmic growth phase when the consumption of NaOH had almost stopped

Influence of Glucose Addition upon Sl O Formation

A 5 liter streptococcal culture was grown under stabilized pH conditions at pH 6 8 To

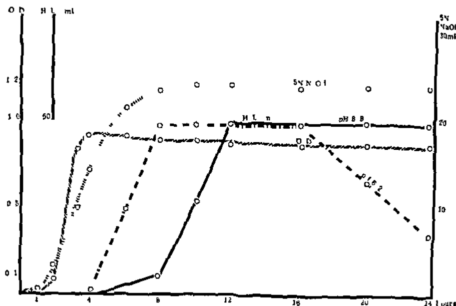


Fig 1 Illustration of changes of O D, consumption of 5N NaOH (ml/l) and hemolytic activity in streptococcal cultures grown under stabilized pH conditions at pH 6 2 and 6 8

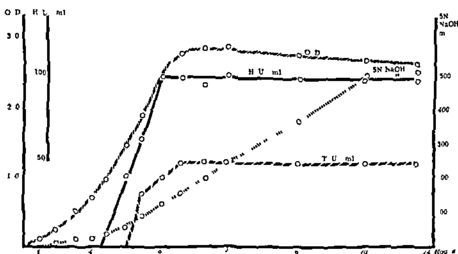


Fig 2 Changes in O D rate of consumption of 5N NaOH (ml/5 l) and hemolytic (H U) as well as combining (T U) activity in a culture grown under stabilized pH conditions at pH 6 8 Glucose was added intermittently to give a final concentration of $\approx$ 1 per cent in the culture during the exponential phase of growth

counterbalance the utilization of glucose—representing the only important energy source for streptococci—260 ml 50 per cent glucose was added intermittently during the first 8 hours in order to maintain the glucose concentration around 1 per cent in the culture

The result of this experiment is illustrated in Fig 2 As may be seen the logarithmic growth phase lasted longer than in the experiments just mentioned which resulted in a higher yield of streptococcal cells—O D almost three times as high as in the experi

it in Fig 1 The consumption of NaOH consequently higher and lasted in pro tion to the longer exponential growth se also longer Within eight hours of ivation the maximal hemolytic activity 0 H U /ml) was reached, that is at the of the logarithmic growth phase and e than 14 hours before the consumption NaOH stopped The determination of ose at different intervals showed that glucose concentration during the first ten rs varied between 8 6–12 0 g/l

igenicity of Sl O Preparations at Different Growth Phases

amples were taken after 6 and 28 hours n the culture described in the last para h After sterile filtration and concentra these concentrated EC materials (EC_6 EC_{28}) were analyzed by immunodiffu and blood agar microchamber technique us a concentrated pool of human sera ASO) and a serum from a sheep hyper iunized with streptococcal products (anti + EC) and compared with an intra ılar streptococcal preparation (IC) As be seen in Figs 3 and 4 the EC_{28} ma d contained a greater number of pre tinogenic factors than the EC_6 material pective of which of the two streptococcal iunsera that was employed in the im iodiffusion analyses Judging from the tion of the precipitation lines the pre tinogens were also present in somewhat ier concentrations in the EC_{28} material nay furthermore be noted that at least of the precipitation bands seen in the s anti IC + EC system coalesced with bands of the IC anti IC + EC pre tation spectrum None of these two lines observed in the EC_6 anti IC + EC iune system The correlation between the s and IC materials in analysis versus SO and anti IC + EC is further illu ted in Fig 5 As may be seen none of the s formed by the IC material and the immunsera corresponded to the pre tates in the zone of inhibition formed in the analysis of EC_{28} material and the same immunsera

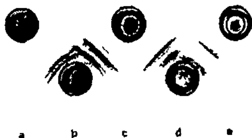


Fig 3 Immunodiffusion pattern obtained by ana lysis of a polyvalent streptococcal hyperimmun serum placed in basins (b) and (d) with intra cellular streptococcal material in the middle basin (c) and compared with EC_6 (a) and EC_{28} (e) (extracellular material taken after 6 and 28 hours of cultivation)



Fig 4 Immunodiffusion analysis of the same an tigen materials as those employed in Fig 3 but analyzed with a concentrated pool of sera from patients with recent streptococcal infection (the two lower basins)

To study the specificity of the hemolytic activity of the EC_6 and EC_{28} materials equal volumes of cholesterol, lecithin, and H ASO were added to aliquots of reduced EC ma terial while another aliquot was oxidized with hydrogen peroxide After incubation at room temperature for two hours the materials were filled into the wells of a blood agar micro chamber and further incubated for 4 hours at room temperature As may be seen in Fig 6 reduced EC_6 and EC_{28} both lyzed the blood

around the well while no hemolysis was registered by cholesterol treated or oxidized EC preparations Cysteine hydrochloride in the same concentration as in the EC preparations

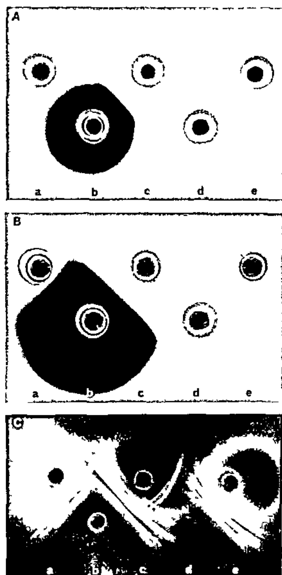


Fig 5 Immunodiffusion analysis in blood agar of a streptococcal hyperimmunserum, basins (a) and (e), and a concentrated pool of patient sera (c) with extracellular (EC_{28}) and intracellular (IC) materials in basins (b) and (d) The development of the clear zone around the EC_{28} basin (b) and the hemolysis inhibition at the position of the SL O—ASO precipitate is illustrated Fig 5 A was taken after 6 hours' of diffusion, B after further 18 hours and C two [illegible] later—after hemolysis of the unaffected erythrocytes by staphylolysin

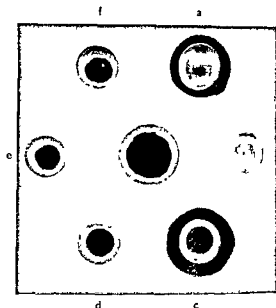


Fig 6 Analysis in blood agar micro chambers of streptococcal preparations (a) and (c) reduced EC_6 and EC_{28}, (f) and (d) oxygenized EC_6 and EC_{28} and (e) and (b) cholesterol treated EC_6 and EC_{28}, respectively The centre well contained only the reducing agent as a control

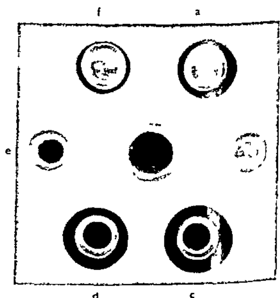


Fig 7 Analysis in blood agar micro chambers of EC_6 + lecithin (a) EC_{28} + H-ASO b) reduced EC_{28} (c) reduced EC_6 (d) EC_6 + cholesterol (e) and EC_6 + H-ASO (f) The centre well contained H-ASO as control

ved no lytic activity nor did H ASO ted twice in saline as may be seen in 7 In this figure it may also be seen that lytic activity of EC_6 was not influenced lecithin but was completely inhibited by lesterol It should further be noted that SO completely neutralized the hemolytic vity of EC ₛ but only partially inhibited hemolytic activity of EC_6

olytic Activity of Sl O Preparations Stored at Different Temperatures

streptolysin O preparation was produced nentioned above under stabilized pH con ns at pH 6 8—the culture being centri d and filtered after 16 hours of growth hemolytic activity of this Sl O prepara was 100 H U /ml which corresponded 0 T U (Todd Units) This material was ensed into forty 10 ml vials which were d at the following temperatures —70°C 0°C —20°C and +4°C At monthly vals during ten months the Sl O prepara was analyzed for hemolytic activity and) combining capacity The activity was d to have decreased less than 10 H U / 5 T U /ml) from that of the original preparation irrespective of the tempera at which the preparation was stored

DISCUSSION

results of the cultivations performed at rent pH levels illustrated that the opti pH for both streptococcal growth and tolysin O formation was between pH nd 7 0 In these cultures the end of the nential growth phase was reached al y within 4 hours of cultivation at which practically no streptolysin O activity l be detected in filtrates of the culture ng the next 4 hours no further increase D of the culture was noted though the mption of NaOH continued and strepto O activity could be detected To ex this discrepancy it is tempting to as that streptolysin O is not actively pro l but rather released by a slow diffusion out of the streptococci at the end of the active growth phase The continued con sumption of NaOH would then represent the hydroxide needed to neutralize the lactic acid produced at the stationary growth phase and the acid material released from the streptococci during the autolysis This is in agreement with results reported by *Fox* (1961) who showed that nonproliferating cell suspensions of streptococci could be sti mulated to synthetize streptolysin O as well as M protein in the presence of radioactively labeled peptides although radioactivity could not be detected in synthetized proteins In these experiments glucose had to be supplied in the reaction mixtures as an energy source The importance of glucose for the production of Sl O was also pointed out by *Bos örmenyi et al* 1967 They found that for strepto coccal growth 0 2 per cent glucose is the lower limiting concentration and that 1 per cent offers optimal conditions both for growth promoting and Sl O formation This was verified in our experiments where an addi tion of glucose prolonged the exponential growth phase resulting in very high bacterial yield and also higher Sl O activity However in this experiment the Sl O formation stopped at the end of the exponential phase and hemolytic activity was demonstrated already in the middle of this phase No further in crease in hemolytic activity was demonstra ble after the end of the logarithmic growth phase These results contradict a passive release from the streptococci as the mecha nism for Sl O formation Several explanations might be discussed Studies on substances with Sl O stimulating effect have been pre sented by several authors *e g Slade & Knox* (1950) *Slade et al* (1951) *Fox (1961)* *Mickelson* (1964) These substances should be present in the growth medium employed in the present study in sufficient concen tration (*Holm & Falsen* 1967) and should not be limiting for the Sl O formation but more detailed studies will be performed on this aspect A *possible mechanism which* could account for the stop in Sl O formation in growing as well as stationary cultures

could be a feed back regulatory system with inhibition of further SLO formation once a certain concentration of SLO is reached. Further studies along these lines are under way.

The discrepancy between the hemolytic activity and the combining activity of the lysin formed during the early exponential phase in the last experiment was studied in detail. The result showed that this lysin had all characteristics of ordinary streptolysin O being hemolytically inactivated by oxidation and by addition of minute amounts of cholesterol but being unaffected by lecithin. However, complete neutralization was not achieved in the presence of antistreptolysin O antibodies added in a concentration which completely inhibited the streptolysin O activity of EC_8. This was highly unexpected as the lytic activity of EC_8 was at least 2.5 times as high as that of EC_6. The immunodiffusion analyses of EC_6 and EC_8 materials showed that at least two additional antigens were present in the EC_{78} material. These two antigens cross reacted with two antigens found in an intracellular streptococcal preparation. None of these two lines corresponded to the SLO-ASO precipitate as analyzed with the method of *Holm & Möller* (1971). It was furthermore noted that the intracellular material—although being rich in precipitinogens—did not show any lytic activity whatsoever.

The experiment performed to study the influence of temperature on the storage of the SLO produced at a constant pH of 6.8 showed that no significant decline could be demonstrated in either lytic or combining activity over 10 months' test period. *Elliott* (1950) described the production of streptococcal proteinase in cultures cultivated at pH 5.5–6.5 and showed that streptococcal proteinase is derived from an inactive precursor which at the end of the growth phase is autocatalytically converted to the active enzyme. We were also able to demonstrate proteinase activity in cultures from streptococci cultivated at pH 6.2 but no activity in the cultures grown at pH 6.8 or above. It is conceivable that streptococcal proteinase might digest already formed SLO and thus being responsible for the gradual loss of hemolytic activity usually observed in SLO batches not produced under stabilized pH conditions. This statement is strengthened by the observation of the decreasing hemolytic activity noted in the streptococcal culture stabilized at pH 6.2 after the end of the logaritmic growth phase.

This study was supported by a grant from "*Statens Medicinska Forskningsråd*", Sweden.

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Acta path microbiol scand Section B 79, 88–94, 1971

SYNERGISTIC EFFECT IN VIRAL-BACTERIAL INFECTION

4 The Leukocyte Response to Parainfluenza Virus Infection in the Respiratory Tract of Mice, and the Influence of Antibiotics on the Development of Infection

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Polymorphonuclear (PMN) leukocyte accumulation in different sections of the respiratory tract of mice is a prominent sign after inoculation with parainfluenza 1 virus Spontaneous invasion of bacteria to the lung, normally present in the upper respiratory tract was frequently observed from the 2 day after viral inoculation Penicillin G, administered in drinking water during the entire course of experiment eliminated the bacteria from the lungs The extent of PMN leukocytosis was also reduced in the lungs and to a lesser extent in the circulating blood The same treatment also reduced significantly the mortality which normally follows the virus infection It is reasonable to believe that a bacterial invasion secondary to the effect of primary virus inoculation is responsible to some extent for the leukocyte accumulation in the respiratory organs, and it may participate in the infectious disease

The inflammatory reaction to the cellular injury produced by viral infections is often a complex one, undergoing a dynamic sequence in the proportion of mononuclear and polymorphonuclear (PMN) cells Also the total number of cells present in the local reaction and in the circulating blood varies widely However, in general, unlike the predominantly PMN leukocytosis in acute bacterial infections the increase of mononuclear cells characterizes the viral infections, especially during the early phases

During previous investigations we have noted a marked accumulation of PMN leukocytes in the trachea epithelium and lamina propria of mice following infection with parainfluenza 1 virus similar to that observed during bacterial infection (4) PMN leukocytes also dominated in the cell mass found frequently in the lumen of the trachea Furthermore, we have noted a bacterial invasion in the lungs by bacteria normally present in the upper respiratory tract The question was raised whether the leukocyte accumulation was to some extent a result of a secondary bacterial invasion Further investigations on the leukocyte response and the possible influence of the secondary bacterial invasion on the development of the disease are the subjects of the present communication To prevent bacterial invasion, antibiotic treatment was employed and the effect of this treat

Received 24 viii 70

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ie results indicate that spontaneous bac-
l invasion secondary to viral infection
have an influence on the development
ie disease and adequate treatment with
iotics may alter the course of disease

[M]ATERIAL AND METHODS

imental animals Young adult HaM/ICR/
Bom albino mice of either sex, weighing 22–
were employed as in previous studies (4)
larly repeated tests for haemagglutinating
and pneumonia virus of mice were all nega

us Parainfluenza 1 virus Sendai strain, as
oyed in previous studies (3, 4), was pro
ed in the allantoic cavity of 9–11 days old
vonated eggs Infectivity titer was titrated in
yonated eggs calculated according to the
od of *Reed* & *Muench* (12) Samples from
ame pool kept at —70° C were employed in
xperiments

cteria Hemophilus influenzae b 51 was used
42 passages on solid medium as in previous
s (4) The preparation of inoculation was
bed in detail earlier

ection of mice All inoculations were done by
ntranasal route under light ether anesthesia
For each experiment mice were selected
mly from a homogenous group All groups
kept under identical conditions

tibiotics Preliminary experiments were con
with Ampicillin injected intraperitoneally
The serum concentration of ampicillin de
d within 48 hours below the minimum in
ry concentration (MIC) of the *H influenzae*
employed Serially repeated i p injections
d in increased mortality therefore this route
ministration was discontinued In the follow
xperiments Penicillin G 8000 IU per ml was
to the drinking water and renewed daily
ge daily water consumption, 5–6 ml was not
nced by the addition of penicillin The daily
of antibiotic averaged roughly 4 million IU
g body weight Serum concentration was
constant at 1 5 μg per ml as measured by
icrotube dilution method (8)

ery and Quantitation of Cells from the Respiratory Tract

e were sacrificed by cervical fracture at
nt times following inoculation The anterior
wall and the pretracheal muscles were dis
away A 14 gauge needle was introduced into the trachea just below the pharynx One ml Hanks balanced salt solution (BSS) containing 1 per cent ethylendiamino tetraacetic acid dipotassium salt (EDTA) and 0 5 IU per ml heparin was forced into the lung and withdrawn after 30 seconds without manipulation with the lung The process was repeated 3 times The mixture so obtained was centrifuged at 600 rpm for 10 minutes The supernatant was streaked out on agar plates containing 5 per cent human blood, and on chocolate plates The pellet was dissolved in 0 1 ml Hanks' BSS for cellular counts

Total cell counts were estimated by a Burker hemocytometer Cells were counted in at least 4 large squares Viability of the cells was established by the trypan blue exclusion method One per cent solution of trypan blue was applied for 5 minutes before counting Differential counts were made on smears stained with Wright's stain At least 100 cells were counted for differential counts and viability tests

The consistency of the cell recovery method was established by comparing the values obtained from 10 healthy mice from the same litter In 8 of the 10 the number and distribution of cells was within ± 10 per cent of the mean value in the other two within ± 25 per cent

Recovery and Quantitation of Cells from the Blood

Samples were obtained from the tail tip Total cell counts were determined by the conventional method in a Burker hemocytometer and the differential counts on Wright stained smears

Statistical methods The results were subjected to statistical analysis employing the Wilcoxon's two sample test for cellular distribution and the χ^2 test for the mortality data

RESULTS

White Cell Response in the Lung Following Parainfluenza Virus Infection

A large group of mice was infected with 10^6 EID_{50} virus At various times after inoculation 6–10 randomly chosen mice were sacrificed and the white cell content of their lungs estimated The number and distribution of white cells at different survival times after inoculation is shown in Fig 1 In normal mice the dominating cell types are mononuclear cells, mostly macrophages and some lymphocytes Only few PMN leukocytes were present in normal animals Following the virus infection a moderate reduction of macrophages and slight increase of PMN leuko

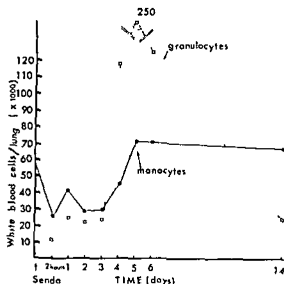


Fig 1 Number of macrophages and PMN leukocytes in the washouts from the lungs of mice at different times after inoculation with parainfluenza 1 Sendai virus

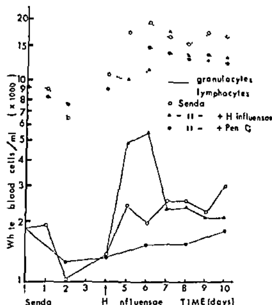


Fig 2 Number of circulating white blood cells obtained at different times after inoculation with parainfluenza 1 Sendai virus and *H influenzae* and during Penicillin G treatment of Sendai virus infected mice

cytes was characteristic during the first few days Total number of cells was slightly reduced From the 4th day the total number of cells increased markedly, mostly due to a more than tenfold increase of PMN leukocytes The highest numbers of cells were obtained on the 5th day following inoculation Fourteen days after inoculation the number of cells and their distribution was practically normal Besides macrophages and PMN leukocytes the majority of other cells were lymphocytes *They were obtained in increasing numbers towards the later phases*

Viability of macrophages did not vary with the time after virus inoculation as determined by the trypan blue exclusion method Between 88 and 95 per cent of these cells were viable It was difficult to obtain reproducible results for PMN leukocytes with the method here employed However, a tendency to obtain more dead cells towards the later phases was clear From normal mice and until 2 days after infection 80 to 95 per cent of PMN leukocytes were viable After the second day the values varied between 60 to 90 per cent

White Blood Cell Response Following Parainfluenza Virus Infection and the Effect of Penicillin G

Six mice were inoculated with 10^6 EID$_{50}$ virus Blood samples were taken daily from the tail tip The daily bleeding did not change the total numbers and the distribution of cells as controlled by serial bleeding of normal mice The number of circulating white blood cells obtained at different times after virus inoculation is shown in Fig 2 Both lymphocytes and PMN leukocytes were reduced during the first two days The reduction was followed by a 3 fold increase of both cell types beginning on the 4th or 5th day The highest values obtained were 1.9×10^3 lymphocytes and 3.1×10^3 PMN leukocytes per ml blood

Another group of 6 mice was inoculated with 10^6 EID$_{50}$ virus and challenged with 2.5×10^7 colony forming unit (CFU) *H influenzae* 4 days later In this group a more marked PMN leukocyte response could be observed with the highest peak at 5.6×10^3 per ml on the 6th day The monocyte

E 1 *Polymorphonuclear Leukocytes and Macrophages Obtained from the Lungs of Parainfluenza 1 Virus Infected Mice with and without Treatment with Penicillin G*

e after ulation	No treatment		Penicillin G treatment	
	PMN leukocytes	Macrophages	PMN leukocytes	Macrophages
) days	2 5×10⁵ (48 5)*	7 1×10⁴ (15 1)	7 1×10⁴ (17 4)§	7 7×10⁴ (25)
) days	ND (19 6)	ND (9 4)	ND (10 4)	ND (19 5)
days	8 0×10⁴ (19 8)	9 0×10⁴ (24 5)	1 8×10⁴ (7 1)§	9 0×10⁴ (32 8)
ormal mice	1 7×10⁴ (3 5)	5 9×10⁴ (57)		

'er cent in parenthes
) Total count not done
, < 0 01

nse was not significantly different from *of virus infected group (Fig 2)*

third group of 6 mice was inoculated 10^6 EID_{50} virus and given Penicillin G ig the whole time of the experiment cellular response was similar to that of above virus infected group The number MN leukocytes obtained was somewhat ı, but the difference was not significant 2)

ence of Penicillin G on the White Cell Response in the Lungs

large number of mice were infected with EID₅₀ virus, and given Penicillin G in drinking water After 5, 10 and 14 days 12 randomly selected mice were sacri and the white cell content of their determined The results are shown in ‿ 1 As in the previous experiment, an ase of the total number of cells and ially the PMN leukocytes was observed extent of leukocytosis was different in als given water and those given anti- s The proportion of PMN leukocytes ned from the Penicillin treated mice significantly reduced on all days, while roportion of macrophages was increased change in proportions was due to a re on of the number of PMN leukocytes ‿ 0 01) The number of macrophages not influenced by the antibiotics

ie leukocytosis which followed the virus tion was not reduced completely by the illin treatment Compared with normal values both the number of macrophages ($p < 0\ 05$) and PMN leukocytes ($p < 0\ 01$) was elevated 5 days after inoculation

Isolation of Bacteria from the Lung "Wash Outs"

At least 10^3 bacteria were isolated from the lungs of 0/10, 1/10, 5/10, 5/10, 6/8 and 7/8 mice on days 1 to 6 after virus inoculation These bacteria were mostly α hemolytic streptococci, corynebacteria and *Staphylococcus albus*, the most usual members of the normal resident flora of the upper respiratory tract of mice No bacteria could be cultured from the wash outs of antibiotic treated mice

Influence of Penicillin G on the Development of Disease

Mice were infected with 10^7 EID_{50} parainfluenza virus One group, (42 mice) was given Penicillin g in the drinking water, the control group (37 mice) received water The cumulative mortality of the two groups is graphically shown in Fig 3 The difference in total mortality after 10 days is highly significant ($p < 0\ 01$), indicating that the antibiotic treatment eliminates one critical factor of disease and cause of death A short antibiotic treatment, lasting 4 days following viral inoculation did not have any reducing effect on the mortality (Fig 4) On the contrary, the penicillin treated group had a higher mortality rate from the 5th day, but the difference is not significant

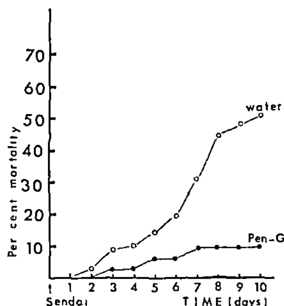


Fig 3 Cumulative mortality of parainfluenza 1 Sendai virus infected mice and the influence of Penicillin G treatment

In the next experiment mice were given 10^6 EID_{50} virus and challenged with *H influenzae*, 2 2 × 10^7 CFU, 4 days later One group (25 mice), was given Penicillin-G, the control group (25 mice) tap water The

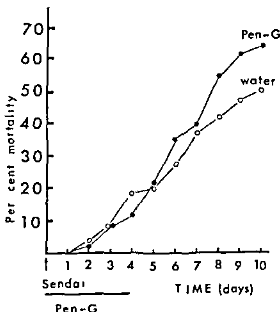


Fig 4 Cumulative mortality of parainfluenza 1 Sendai virus infected mice and the influence of a *short course of Penicillin G treatment*

cumulative *mortality of these groups during* 10 days observation time is shown in Fig 5 The mortality seems to be reduced in the antibiotic treated group, however, the difference is not significant The treatment increased the length of survival after infection Mean survival time of the untreated group *6 56 days, was increased to 7 92* days in the treated group ($p < 0\ 05$)

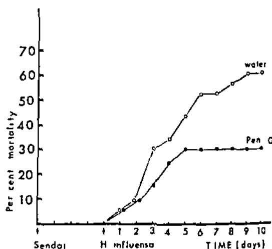


Fig 5 Cumulative mortality of parainfluenza 1, Sendai virus and *H influenzae* infected mice, and the influence of Penicillin G treatment

DISCUSSION

The primary question of this study was whether the leukocytosis in the respiratory *tract following virus inoculation is influenced* by a bacterial invasion secondary to the virus inoculation The data confirms our previous findings (4), that following virus infection there is a marked accumulation of leukocytes predominantly polymorphonuclears The cell increase is initiated after a 3 day long lag *period under which only small changes* could be observed This is compatible with the data of *Francis & Stuart-Harris* (7) who studied the effect of influenza on the ferret respiratory tract Similar observations were made during parainfluenza 3 virus infection in hamsters (2) *In serial pathological examination of the lungs these authors observed* PMN leukocyte invasion from the 3d day increasing to the 5th day after virus inoculation

ie present data suggest that antibiotics ce the PMN leukocyte accumulation illin treatment has also eliminated the ria from the lungs It should be men- d that bacteria isolated from the respi- y tract of the mouse strain here em- d were overwhelmingly sensitive to Pe in G *Doloway & Moldoon (5) could* detect PMN leukocyte aggregates in the sections of influenza A virus infected free mice, were the normal flora' of ratory tract does not exist In contrast, , from conventional mice contained large bers of PMN leukocytes and some bac after infection with the same influenza 1 *Buthala & Soret* (2) on the other side I not isolate any bacteria or Mycoplasma lungs of parainfluenza 3 infected ham where the invasion of leukocytes was d No significant alteration of the histo ological appearance could be observed administration of antibiotics In the nt study the number of PMN leuko obtained after viral inoculation was also ficantly elevated even during continous ment with penicillin, as compared with ated controls It seems reasonable that rtain leukocyte invasion is normal as a t response to the virus infection without econdary bacterial invasion This leuko is is probably to a large extent a reac to the cell destruction products

ie circulating white blood cells were little nced by virus infection in mice given iotic treatment The response to virus tion was similar in mice without anti *s, but the PMN leukocyte response was* what more pronounced towards the later :s of infection In contrast, marked PMN cytosis could be observed when second nfection with *H influenzae* was given ocyte response was essentially the same l three groups The similarity of mono ar and the dissimilarity of PMN leuko- *response in* the three groups is contrast The extent of PMN leukocytosis in the ' corresponds clearly *with the extent of* nal incasion in the respiratory tract

· normal distribution of white blood cells in mice is markedly different from the human values (14), which makes a direct comparison with leukocyte response to human infection difficult However, it is interesting that the leukocyte response after viral infection in the human respiratory tract does not follow any defined pattern, and the variation *has no clear relation to the particular* infectious agent (6, 11) These authors suggest that neutrophilia is not specific for any particular virus but rather the character of host response Besides tissue destruction and release of products of this destruction, secondary bacterial invasion might be a plausible explanation Infection with virus alone, without further progress of disease, is in sufficient to call for the response (11) *Parrott et al* (10) reported some relationship between the presence of elevated white cell count and the coexistence of pathogenic bacteria in the oropharynx of virus infected pa tients The present findings are in good correlation with these reports

The antibiotics given for the entire course of experiment altered the outcome of dis ease by reducing the mortality The extent of this reduction was in fact surprisingly large as it is not in accordance with our present concept of the pathogenetic role of resident bacterial flora *Bowden et al* (1) reported recently that chlortetracycline, 2 g per liter, added to the drinking water reduced the mortality of X irradiated mice from 70 per cent to nil It is reasonable to believe that the route of action is through a reduction of the bacterial flora, which is the *source of the secondary invasion when the* host defence factors are weakened The bac terial contamination in the virus infected lungs was in fact eliminated completely in the penicillin treated mice The effect was less pronounced when *H influenzae* was given as secondary challenge The difference might be due to insufficient concentration of penicillin in the lungs The MIC of Peni cillin-G on this organism is only slightly be *low the concentration measured in the blood*

No reference was available on experimen tal investigation on the effect of antibiotics

on viral respiratory tract infection Clinical reports were generally negative concerning this question (9, 13, 15, 16, 17) The timing and length of treatment is poorly specified in most of these studies As the present data show it is important that the antibiotics are given for sufficient length of time in adequate doses When ceased prematurely the effect might be adverse

At the present moment there is no clear explanation for the apparently dissimilar effect of antibiotics in human viral respiratory tract infections and the present para influenza mice model It is possible that differences between the bacterial flora of human and mouse respiratory tract can be a part of explanation Development of resistant strains and dominating gram negative bacterial flora, often seen in human disease, was not observed in the present study

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CHEMICAL ANALYSIS OF A SALIVARY GLYCOPROTEIN WITH BLOOD-GROUP SUBSTANCE- AND VIRUS INHIBITION ACTIVITIES

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A human salivary glycoprotein which exhibits blood group substance activity in the ABO and Lewis systems, and which also inhibits the haemagglutinins of influenza virus (*Rolla & Jonsen* 1968) has been analysed Glycoprotein preparations from a non secretor (O, Le a b) and from a secretor (O Le a b⁺) showed very similar amino acid composition, indicating identical protein cores in the two glycoproteins, whereas the secretor preparation contained more amino sugars and fucose The salivary blood group active glycoproteins had an amino acid composition different from the ovarian cyst blood group substances and contained more protein than these preparations

ary glycoproteins cover the oral mucosa, ingiva and the teeth, and have been ied several roles in oral physiology and ology (*Chernick & Barbero* 1963, *Dische Leach* 1964, *Watkins* 1966, *Jenkins*) The purification procedure of a hu salivary glycoprotein possessing blood- substance (ABO, Le) activity, and also inhibits the haemagglutination of nza virus has been reported previously *la & Jonsen* 1968) The glycoprotein ration was homogeneous in the ultra- fuge, in iso-electric focussing and in disc- ophoresis It contained about 50 per carbohydrate including a large compo of neutral sugar, had a molecular weight)2 000, and a very low pI (< 3) The

In the present communication the amino acid analysis of the glycoprotein is reported, and also the identification and quantitation of neutral sugars Glycoprotein preparations of blood group substance activity O, Le a⁺, b (non-secretor) and O, Le a⁻, b⁺ (secretor) were analysed

MATERIALS AND METHODS

Preparation of Glycoproteins

Saliva was collected from two donors possessing difference blood group activity in their saliva (O Le a⁺, b and O Le a , b⁺) Sampling of saliva,

... previously (Rolla 1967, Rolla & Jonsen 1968)

Protein Hydrolysis

Samples for hydrolysis were dried to constant weight at 60° and suspended in sufficient 6 N HCl to give samples containing 1 mg glycoprotein per ml acid The following procedure was adopted be fore hydrolysis, to evacuate the ampoules

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A flask containing purified nitrogen[1] and a Speedivac Centrifugal Freeze Dryer were connected so that the ampoules could be filled with nitrogen and evacuated alternatively After freezing in solid CO_2 and acetone the ampoules were three times alternatively filled with nitrogen and evacuated to 0 2 mm Hg Air trapped in the acid was partly extracted by melting the acid under vacuum until small bubbles were formed The ampoules were sealed under vacuum by melting the necks of the ampoules

The glycoprotein possessing Le a⁺ b blood group activity was hydrolysed for 24–48–72–84 and 96 hours The glycoprotein with Le a b blood group activity was hydrolysed 24–48 and 72 hours

After hydrolysis the necks of the ampoules were broken and the contents lyophilized The residues after lyophilization were dissolved in 1 ml distilled water to which was added 0 1 μ mol nor leucine as internal standard for the amino acid analysis

Sugar Hydrolysis

Samples of 1 mg glycoprotein were added to 3–4 ml 1 5 N HCl and hydrolyzed for 1, 2, and 3 hours at 105° After hydrolysis the samples were evaporated to dryness and redissolved in distilled water Before analysis each sample was filtered, using a Sartorius Filtration Apparatus MD 50 15, with a Gottingen membrane filter Lsg 60 The membrane had an average pore size of 5 mμ and an exclusion limit of 10 000 The filtration procedure has previously been described (*Sonju* 1969) To each sample was added 50 μg ribose as internal standard before the analysis

Amino Acid Analyses

Amino acid analyses were done with a Technicon AutoAnalyzer using the standard procedure (Technicon's AutoAnalyzers Handbook) and Chromobead Type B resin

For the analyses of the glycoprotein possessing Le a b⁺ blood group activity, the pH of the elution buffers was lowered by 2 3 to improve the resolution of methionine and cystine

Substances eluted at positions different from those of the standard amino acids[2] (asp, thr, ser glu pro gly, ala val, cys, met, ile, leu, tyr, phe, lys, his, arg) were identified by their relative elution positions and by enrichments of the hydrolysates with individual amino acids The following amino acids and amino sugars were added as enrichment in various hydrolysates to ascertain elution positions methionine, valine, galactosamine and cystine The elution positions of cysteic acid and methionine sulphoxide have previously been ascertained by discrete sampling and paper chromatography (*Sonju* 1969)

Sugar Analyses

The neutral sugars were analysed on the Technicon AutoAnalyzer using a procedure described by Technicon (Technicon Sugar Chromatography) and discussed by *Kesler* (1967) The sugars were eluted from a water jacketed column 3 × 750 mm containing Chromobead type S resin and mixed with orcinol/sulphuric acid The optical density of the coloured complex was read at 420 mμ and recorded by a multipoint recorder The elution positions of the various sugars were ascertained by analysis of samples containing one or two different sugars until the elution positions of ribose, mannose fucose, galactose and glucose had been found

Quantitative Determinations

Amino acids A Technicon Integrator/Calculator Model AAG was used for quantitative determinations Colour factors necessary for the calculations [illegible] of the Standard Ami[illegible] tion was [illegible] acids and [illegible] sulphoxide galactosamine and glucosamine, respectively

Neutral sugars Quantitations were done as described for the amino acids Colour factors were obtained from analysis of a solution containing 50 μg each of the following sugars ribose mannose fucose galactose and glucose

RESULTS

Amino Acid Analysis

Substances eluted at positions different from those of the standard amino acids, were identified as cysteic acid, methionine sulphoxide, galactosamine and glucosamine

The amounts of cysteic acid and methionine sulphoxide varied from one analysis to another and have been added to the amounts of cystine plus methionine in Tables 1 and 2

The other ninhydrin reactive substances had elution positions identical to those of the standard amino acids The small peak representing cystine + methionine (Fig 1) was quantitated by the use of the mean colour factor of the two amino acids

The decrease of pH in the second series of analysis separated the two peaks representing cystine and methionine

[1] Norsk Hydro, Rjukan Norway

[2] Technicon Standard Amino Acid Solution

TABLE 1 *Results of Amino Acid Analysis of 1 mg Glycoprotein at Various Times of Hydrolysis*

Amino acids and amino sugars	Le a⁺b Hours of hydrolysis					Le a⁻b⁺ Hours of hydrolysis		
	24	48	72	84	96	24	48	72
Cys ac	0.06							
Asp	0.29	0.32	0.36	0.34	0.34	0.27	0.27	0.26
Thr	0.20	0.21	0.21	0.19	0.20	0.29	0.28	0.27
Ser	0.20	0.21	0.21	0.20	0.20	0.23	0.21	0.19
Glu	0.24	0.28	0.28	0.26	0.27	0.27	0.26	0.26
Pro	0.15	0.19	0.15	0.18	0.18	0.19	0.18	0.20
Gly	0.23	0.26	0.26	0.25	0.25	0.25	0.25	0.26
Ala	0.18	0.21	0.20	0.20	0.20	0.23	0.22	0.22
Val	0.18	0.23	0.22	0.22	0.22	0.18	0.18	0.17
Cys +Met	0.02	0.03	0.05	0.03	0.02	0.03	0.03	0.03
Ile	0.09	0.12	0.11	0.11	0.11	0.10	0.10	0.09
Leu	0.19	0.23	0.22	0.21	0.22	0.17	0.17	0.17
Tyr	0.09	0.11	0.11	0.10	0.10	0.10	0.11	0.10
Phe	0.09	0.11	0.12	0.10	0.11	0.09	0.10	0.12
Lys	0.10	0.12	0.12	0.11	0.11	0.11	0.10	0.12
His	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Arg	0.11	0.13	0.13	0.13	0.13	0.12	0.11	0.12
Glu am	0.12	0.11	0.07	0.06	0.06	0.43	0.26	0.11
Gal am	0.04	0.03	0.02		0.02	0.18	0.13	0.09

μ mol amino acids per mg glycoprotein

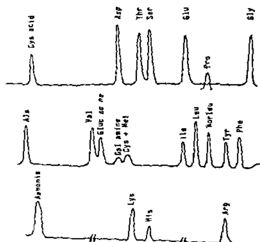


Fig. 1. Elution diagram of amino acids 24 hour hydrolysate of a sublingual glycoprotein.

The results of the amino acid analysis are in Table 1 given as μ mol amino acids recovered at the various times of hydrolysis. The table demonstrates a general increase in amino acids from 24–48 hours of hydrolysis for the Le a⁺b glycoprotein. After 48 hours the amounts of the stable amino acids (asp, pro, glu, gly, ala, val, ile, leu, tyr, phe, lys his, arg) reached a nearly constant level, while the amounts of the hydroxy amino acids decreased in amounts. The increase of threonine and serine from 24 to 48 hours of hydrolysis was unexpected as these amino acids are among the earliest residues to be released (*Desnuelle* & *Casal* 1948). A continuous destruction of these amino acids in hot acid solutions is also reported (*Rees* 1946, *Pusztai* & *Morgan* 1963). The slow release of the hydroxy amino acids, combined with the general increase in amounts of the more stable amino acids, could indicate an incomplete hydrolysis after 24 hours. But as no peptides were detected, and as the analysis of the Le a⁻b⁺ glycoprotein showed a continuous decrease in amounts of threonine and serine, the results of the 24 hour hydrolysate were excluded in calculations of the mean

TABLE 2 *Amino Acid Composition of a Sublingual Glycoprotein from Two Individuals with Different Blood Groups in the Lewis System*

Amino acids amino sugars		Le a⁺ b	Le a b⁺
Asp	m	12 1	10 1
Thr	h	7 5	10 9
Ser	h	7 5	8 6
Glu	m	10 0	10 1
Pro	m	6 4	7 1
Gly	m	9 3	9 4
Ala	m	7 1	8 2
Val	m	7 9	6 7
Cys + Met	h	1 8	1 1
Ile	m	3 9	3 8
Leu	m	7 9	6 4
Tyr	m	3 9	3 8
Phe	m	3 9	3 8
Lys	m	4 3	4 1
His	m	1 8	1 9
Arg	m	4 6	4 1
Glu	am e	5 4	21 7
Gal	am e	2 1	8 6

Mol per 100 mol amino acids (amino sugars not included in the total)
m = obtained from mean values of Table 1
h = obtained from the highest value in Table 1
e — obtained from T_0 values

amounts of the different amino acids in Table 2

The amounts of the amino sugars in both series of analysis decreased considerably with increasing times of hydrolysis The amounts given in Table 2 were found by extrapolating their degradation curves to T_0 values

In Table 2 the amino acid composition of the sublingual glycoprotein from the two individuals are given as mol per 100 mol amino acids In calculating the mol percentage the amino sugars were excluded in order to obtain a better comparison of the amino acid composition of the two glycoproteins

The amounts of the stable amino acids are calculated from the mean of the amounts found at the varies times of hydrolysis (except the 24 hour hydrolysate of Le a⁺ b)

For the hydroxy and sulphur containing amino acids the highest amount obtained was used

The most characteristic features of the amino acid composition were high amounts of acidic amino acids and small amounts of sulphur containing and basic amino acids

The only striking difference between the glycoproteins obtained from the two individuals was a four times increase in amino sugars in the Le a b⁺ The high amounts of amino sugars was accompanied by a slight increase of threonine

Sugar Analysis

Several unidentified substances were present in all hydrolysates and were all eluted during the first 3½ hours (Fig 2) Ribose (internal standard) mannose, fucose galactose and glucose were fairly well separated and occupied the same elution positions in all chromatograms

The amounts of the individual sugars were found to decrease very rapidly with increasing times of hydrolysis The T_0 amounts given in Table 3 were found by extrapolation of the degradation curves

No differences in T_0 amounts of mannose galactose and glucose were found The amount of fucose was approximately doubled in the glycoprotein possessing Le a b⁺ activity In addition to the higher amount of fucose differences were also evident in the

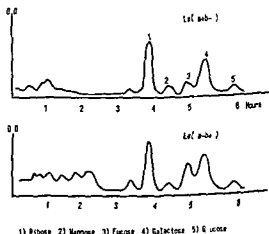


Fig 2 Elution diagram of neutral sugars 1 hour hydrolysate of a sublingual glycoprotein (ribose internal standard)

TABLE 3 *Carbohydrate Analysis of 1 mg Glycoprotein*

	Le a⁺ b				Le a b⁺			
	Hours of hydrolysis				Hours of hydrolysis			
	1	2	3	T_0	1	2	3	T_0
Mannose	3 1	1 5	1 0	4 0	4 5	4 5	3 6	5 0
Fucose	12 0	10 4	5 8	17 0	32 2	27 7	22 7	36 0
Galactose	36 9	27 3	14 0	48 0	39 2	31 6	26 4	47 0
Glucose	18 8	7 1	3 4	12 0	8 1	5 9	4 8	12 0

Amounts in μg (hydrolysis in 1 5 N HCl)

amounts of unidentified substances present in the two glycoproteins The relative amounts of mannose, fucose, galactose and glucose were 1 4 12 3 and 1 9 12 3 in the non secretor and secretor preparations, re spectively

Pilot experiments showed that the haem-agglutination inhibition activity against virus was alkali stable (in 0 5 N NaOH and 0 3 M sodium borohydride for 192 hours at room temperature) Amino acid analyses of alkali treated, dialyzed samples showed no or neg-ligible loss of threonine serine and amino sugars

DISCUSSION

The present results, indicating about 40 per cent of amino acids, are in good agreement with the data obtained by Folin tests on in tact glycoprotein preparations (*Rølla & Jon sen* 1968) The recovery of neutral sugars from the hydrolysates, however, was signifi cantly lower than that obtained by non specific methods used previously Our hydro lysis conditions may have been too mild or this low yield may have been caused by inter-action of reducing sugars with amino sugars during hydrolysis and the presence of neutral sugars linked to N acetylhexosamines (*Gott schalk* 1963, *Neuberger & Marshall* 1966) However the sugar analyses have interest as qualitative identification of the neutral sug ars of the glycoprotein, and give also data on the relative amounts of these sugars The chemical composition of the two glycoprotein preparations shows that their amino acid com-position was essentially the same The small discrepancies in amino acid composition may be ascribed to somewhat different conditions during hydrolysis due to the higher content of carbohydrate in the secretor preparations This indicates a protein core common for the two glycoproteins This concept is also con-firmed by the finding that saliva from secre tors and non-secretors exhibited the same pattern in disc-electrophoresis (*Caldwell* 1969)

The preliminary observation that the carbo hydrate protein linkage was alkali stable seems to exclude an O glycosidic linkage between the carbohydrate residues and the polypeptide chains The moderate amounts of OH amino acids point in the same direction The high amounts of aspartic acid in our prepara-tions indicate the amide group of asparagine as a likely point of attachment between the carbohydrate residues and the protein

The differences in the two glycoprotein preparations (shown by their biological ac-tivities) seem to rest in the carbohydrate side chains where quantitative differences were evident Both the glycoprotein prepara-tions, however, exhibited haemagglutination inhibition against virus, presumably because of the presence of terminal sialic acid in both

Bound glucose could be found in all the hydrolysates, although this is a neutral sugar which only appears occasionally in glyco-proteins (*Neuberger et al* 1966)

Among the salivary glycoproteins so far isolated, bovine- (BSM), ovine- (OSM), and

porcine submaxillary glycoproteins are best known These glycoproteins are all prepared from gland extracts, and are not necessarily present in the saliva Two different types of BSM and OSM have been isolated, one major type being most abundant, and a minor type accounting for 5–7 per cent of the total (*Pigman & Tettamanti* 1968) The amino acid composition of BSM and OSM major types shows that threonine, serine and glycine are the main amino acids, followed by alanine and proline These amino acids accounts for about 80 per cent of the total amino acid content The carbohydrate part of the molecules consists of galactoseamine and sialic acid, linked to the protein core by O glycosidic linkages, and very small amounts of neutral sugars are found These characteristics are not shared by our glycoprotein, where the only amino acid which represents more than 10 per cent of the total is aspartic acid, and which contains relatively high amounts of neutral sugars A certain similarity to the BSM minor can be observed, which also contains high amounts of acidic amino acids and small amounts of basic amino acids It is interesting to note that both BSM minor and our glycoprotein are strongly adsorbed to hydroxylapatite (*Pigman & Tettamanti* 1968, *Rolla* & *Mathiesen* 1970) Our salivary glycoprotein preparation has an amino acid composition more like the *Tamm* and *Horsfall* urinary glycoprotein (*Maxfield* 1966) and the alfa$_1$—acid glycoprotein from human serum (*Jeanloz* 1966)) In both these glycoproteins the glucosamine is the main amino sugar The amino acid composition of blood-group substances purified from ovarian cysts is different from our substances, and they contain less amino acids than our preparation (7 to 26 per cent compared with 40 per cent)

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Acta path microbiol scand Section B 79 102 106 1971

FLAVOBACTERIUM MENINGOSEPTICUM ISOLATED FROM THE GENITALS

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Flavobacterium meningosepticum has been shown to be the cause of hospital infections in the form of meningitis in premature infants This study reports the result of a search for this micro-organism among specimens submitted for examination for *Neisseria gonorrhoeae* Out of 27,600 specimens examined 88 strains of *F meningosepticum* were isolated the majority originating from females There were no characteristic clinical findings and no specific symptoms in the patients It is concluded that the female genitals must be considered as possible source of hospital infection with *F meningosepticum*

Flavobacterium meningosepticum is an aerobic, gram negative, non motile rod, causing hospital infections in the form of meningitis in premature infants (for review, see *Olsen 1969*) (11) and bacteraemia in adults (11) The micro organism has been demonstrated in the nose and throat of healthy newborn infants (1, 3, 13), in hospital environments (3, 11), and in medicaments (11, 12) It has also been isolated from soil and stream water (11) *F meningosepticum* has seldom been isolated from adults (6, 9)

In the present study a search was made for *F meningosepticum* among specimens from the genitals in an attempt to ascertain a possible source of infection in connection with the hospital infections in premature infants

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MATERIAL AND METHODS

The material consists of unselected specimens submitted by general practitioners, medical officers of health venereologists and hospitals for examination for *N gonorrheae* The total number examined was 27 600

Inoculation was made on to chocolate agar plates (10) containing heated horse blood instead of haemoglobin Medium without the addition of antibiotics and medium containing 25 units/ml polymyxin B + 2 µg/ml vancomycin + 25 units/ml mycostatin were employed The plates were incubated for 1 2 days at 36° C in an atmosphere containing 10 per cent carbon dioxide

Biochemical Properties

On the basis of previous investigations (6 11) *F meningosepticum* can be characterized by the following properties

1 It is a gram negative non motile rod there is no growth at 42° C it is oxidase positive and catalase positive

2 Indole is produced and gelatine liquefied nitrates are not reduced there is no or only weak splitting of urea

3 No growth occurs in a mineral medium with ammonium sulphate as nitrogen source and acetate or butyrate or lactate as only source of carbon

4 Oxidative acid production (Hugh & Leifson O/F medium) (4) was estimated by analysis of 185 strains isolated in a previous study(11), all of which possessed the properties mentioned in points 1–3 These strains were isolated from specimens from patients, hospital environments, soil, and stream water On this basis the following pattern was obtained

Glucose	(99)	+	Xylose	(3)	–
Levulose	(88)	d	Galactose	(2)	–
Maltose	(99)	+	Rhamnose	(1)	–
Trehalose	(94)	+	Raffinose	(1)	–
Lactose	(32)	d	Inulin	(0)	–
Sucrose	(29)	d	Salicin	(0)	–
Glycerol	(96)	+	Adonitol	(0)	
Mannitol	(86)	d	Dulcitol	(0)	–
Ethanol	(57)	d	Sorbitol	(0)	–
Starch	(38)	d	Inositol	(0)	–
l arabinose	(3)	–			

The figures in brackets indicate the percentage of positive reactions + indicates 90 to 100 per cent strains positive d 11 to 89 per cent positive – 0 to 10 per cent positive

This carbohydrate pattern was also found in eight strains which differed as regards the properties mentioned in point 2, in that three reduced nitrate beyond nitrite and one reduced nitrate into nitrite Two did not produce indole and a further two either produced indole or liquefied gelatine (11)

The media and methods used were as described by *Jessen* (5) However, indole production was examined after extraction of indole by shaking with xylene before the addition of Ehrlich Bohme s reagent The ONPG reaction was performed as described by *Bulow* (2) *F meningosepticum* has not been examined previously by this test

The gelatine tubes were incubated at 22° C and the remaining tubes at 30° C

The mineral media were observed for 6 days and growth at 42° C (in semi solid agar) for 4 days Nitrate and indole tubes were incubated for 2 days The Hugh & Leifson and gelatine tubes were observed for 30 days A yellow colour in the Hugh Leifson tubes was recorded as positive result a dark blue colour indicating alkalization as negative and the remainder as weakly positive

Sensitivity to Antibiotics

Sensitivity to antibiotics was determined by the agar diffusion method using Sensitabs® (Rosco) It is known from a previous investigation (11) that using this method *F meningosepticum* has the following sensitivity pattern

Erythromycin	+ + +, + +
Chloramphenicol	+ +, +
Streptomycin	+ +, +
Tetracycline	+
Kanamycin	+, 0
Penicillin	0
Sulphamethizole	0
Colimycin	0

In Table 2 + + +, + + are recorded as sensitive and +, 0 as resistant

Method of Isolation

All colonies showing yellow pigmentation on the primary plates and all colonies morphologically resembling *F meningosepticum* were examined by the oxidase test Pure cultures of oxidase positive colonies were obtained on 5 per cent blood agar plates incubated for 2 days at 30° C and examined for indole production and mode of growth in semi solid agar The indole positive, aerobic, non motile strains were inoculated on to agar plates, from which all the above mentioned tests were performed The oxidase test was repeated

RESULTS

Out of a total of 27,600 specimens, 114 strains of *F meningosepticum* were isolated from 90 patients The distribution of sex and age of the patients is shown in Table 1

TABLE 1 Flavobacterium Meningosepticum *Isolated from the Genitals Age and Sex Distribution*

	Age groups (years)					
	14 20	21 30	31–40	41 50	51–55	Total
Female	24	40	9	3	1	77
Male	1	11	1	0	0	13
Total	25	51	10	3	1	90

Further information was obtained concerning 81 patients, 70 of of whom were women *N gonorrhoeae* infection was diagnosed in 23 females, 40 had vaginal discharge, 15 cervical erosin, and three vaginitis *Trichomonas vaginalis* was demonstrated in three patients, and three were pregnant Five out of the 11 males were under treatment for

TABLE 2 *Flavobacterium meningosepticum*

Group no	No of strains	Yellow colour	Nitrate	ONPG	Growth at 42° C	Glucose	Levulose	Maltose	Trehalose	Lactose	Sucrose	Glycerol	Mannitol	[illegible]
Properties of *F meningo septicum*		d	–	?	–	+	d	+	+	d	d	+	d	d
1	12	0	0	0	12	0	0	0	0	0	0	0	0	0
2	24	18	0	0	0	7 17w	0	24	0	0	0	0	0	0
3	24	12	0	12	0	7 17w	0	24	0	0	0	24	0	0
4	2	0	0	0	0	2	0	2	0	0	0	0	0	2
5	15	9	3	3	0	12 3w	0	15	0	0	0	15	0	15
6	5	3	2	0	0	5	0	5	0	0	5	5	0	5
7	?	2	0	0	0	2	0	2	2	0	0	0	0	0
8	2	2	0	1	0	2	0	2	2	0	0	0	0	0
9	1	0	0	1	0	1	0	1	1	0	0	1	0	1
10	2	2	2	0	0	2	0	2	2	0	0	2	0	1
11	1	1	0	0	0	1	1	1	1	0	1	1	0	0
12	2	1	1	0	0	2	2	2	2	0	0	2	0	2
13	1	1	0	0	0	1	1	1	1	0	1	1w	1w	1
14	2	1	0	1	0	2	2	2	2	0	0	2	0	?
15	3	3	0	2	0	3	2	3	3	0	0	0	0	3
16	1	0	0	0	0	1	1	1	1	1	1L	1	0	0
17	1	1	0	1	0	1	1	1	0	0	0	1	0	1
18	4	4	4	1	0	4	4	4	0	0	0	4	0	4
19	1	1	0	1	0	1	1	1	0	0	0	1	0	1
20	1	0	0	1	0	1	1	1	0	1	1	1	0	1
21	2	2	2	2	0	2	2	2	2	0	2	2	0	2
22	4	4	3	1	0	4	4	4	0	0	4	4	0	4
23	2	2	0	2	0	2	2	2	0	0	2	0	0	?
Total	114	69	17	29	12	65 37w	24 1w	102	19	2	16 1L	66 1w	1w	49

Figures indicate the number of strains giving positive reactions in the tests concerned As regards anti biotics the figures indicate the number of strains giving + + + or + + reactions to the antibiotics in question w – weak reaction L = positive reaction later than 1 week d – variable reaction R = resistant to the antibiotic, S – sensitive to the antibiotic positive nitrate reduction beyond nitrite

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	L arabinose	Xylose	Rhamnose	Galactose	Raffinose	Inulin	Salicin	Sulpha methizole	Penicillin	Tetracycline	Streptomycin	Chloram phenicol	Erythromycin	Colimycin	Kanamycin
	–	–	–	–	–	–	–	R	R	R	d	d	S	R	R
	0	0	0	0	0	0	0	3	12	11	11	12	12	11	1
	0	0	0	0	0	0	0	0	0	10	8	24	21	0	0
	0	0	0	0	0	0	0	0	0	13	14	18	21	0	0
	0	0	0	0	0	0	0	0	0	1	2	2	2	0	0
	0	0	0	0	0	0	0	0	0	12	13	13	11	0	1
	0	0	0	0	0	0	0	0	0	4	2	0	2	0	0
	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
	0	0	2	0	0	0	0	0	0	2	2	0	2	0	0
	0	0	1w	0	0	1L	0	0	0	1	1	0	1	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1w	1w	0	0	0	0	0	0	0	0	0	0	1	0	0
	0	0	2	0	0	0	0	1	0	1	2	0	2	0	0
	0	3w	3w	0	0	0	0	0	0	1	3	0	2	0	0
	0	1w	0	1	1L	0	1w	0	0	1	0	1	0	0	0
	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0
	4	0	0	0	0	0	4	0	0	3	4	3	1	0	0
	1	1	0	0	0	0	1	0	0	1	1	1	1	0	0
	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0
	1	0	0	1	2	2	2	0	0	2	2	2	2	0	0
	4	0	0	0	4	4	4	1	0	3	4	3	3	0	0
	2	2	0	2	0	2	2	1	0	2	2	2	2	1	1
	12 1w	4 5w	4 4w	5	7 1L	9 1L	14 1w	6	12	71	74	84	88	12	3

In addition to the properties shown all the strains were gram negative rods, non motile, aerobic, oxidase positive, catalase positive and indole positive, and all liquefied gelatine None of the strains produced acid from adonitol dulcitol sorbitol and inositol None grew with acetate, lactate, and butyrate as only source of carbon

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Group no	No of strains	Yellow colour	Nitrate	ONPG	Growth at 42° C	Glucose	Levulose	Maltose	Trehalose	Lactose	Sucrose	Glycerol	Mannitol
Properties of *F meningo septicum*		d		?	–	+	d	+	+	d	d	+	d
1	12	0	0	0	12	0	0	0	0	0	0	0	0
2	24	18	0	0	0	7 17w	0	24	0	0	0	0	0
3	24	12	0	12	0	7 17w	0	24	0	0	0	24	0
4	2	0	0	0	0	2	0	2	0	0	0	0	0
5	15	9	3	3	0	12 3w	0	15	0	0	0	15	0
6	5	3	2	0	0	5	0	5	0	0	5	5	0
7	2	2	0	0	0	2	0	2	2	0	0	0	0
8	2	2	0	1	0	2	0	2	2	0	0	0	0
9	1	0	0	1	0	1	0	1	1	0	0	1	0
10	2	2	2	0	0	2	0	2	2	0	0	2	0
11	1	1	0	0	0	1	1	1	1	0	1	1	0
12	2	1	1	0	0	2	2	2	2	0	0	2	0
13	1	1	0	0	0	1	1	1	1	0	1	1w	1w
14	2	1	0	1	0	2	2	2	2	0	0	2	0
15	3	3	0	2	0	3	2	3	3	0	0	0	0
16	1	0	0	0	0	1	1	1	1	1	1L	1	0
17	1	1	0	1	0	1	1	1	0	0	0	1	0
18	4	4	4	1	0	4	4	4	0	0	0	4	0
19	1	1	0	1	0	1	1	1	0	0	0	1	0
20	1	0	0	1	0	1	1	1	0	1	1	1	0
21	2	2	2	2	0	2	2	2	2	0	2	2	0
22	4	4	3	1	0	4	4	4	0	0	4	4	0
23	2	2	0	2	0	2	2	2	0	0	2	0	0
Total	114	69	17	29	12	65 37w	24 1w	102	19	2	16 1L	66 1w	1w

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	–		–		–	–		R	R	R	d	d	S	R	R
	0	0	0	0	0	0	0	3	12	11	11	12	12	11	1
	0	0	0	0	0	0	0	0	0	10	8	24	21	0	0
	0	0	0	0	0	0	0	0	0	13	14	18	21	0	0
	0	0	0	0	0	0	0	0	0	1	2	2	2	0	0
)	0	0	0	0	0	0	0	0	0	12	13	13	11	0	1
>	0	0	0	0	0	0	0	0	0	4	2	0	2	0	0
2	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
2	0	0	2	0	0	0	0	0	0	2	2	0	2	0	0
1	0	0	1w	0	0	1L	0	0	0	1	1	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	1w	1w	0	0	0	0	0	0	0	0	0	0	1	0	0
	0	0	2	0	0	0	0	1	0	1	2	0	2	0	0
	0	3w	3w	0	0	0	0	0	0	1	3	0	2	0	0
	0	1w	0	1	1L	0	1w	0	0	1	0	1	0	0	0
	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0
	4	0	0	0	0	0	4	0	0	3	4	3	1	0	0
	1	1	0	0	0	0	1	0	0	1	1	1	1	0	0
	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0
!	1	0	0	1	2	2	2	0	0	2	2	2	2	0	0
I	4	0	0	0	4	4	4	1	0	3	4	3	3	0	0
!	2	2	0	2	0	2	2	1	0	2	2	2	2	1	1
8	12 1w	4 5w	4 4w	5	7 1L	9 1L	14 1w	6	12	71	74	84	88	12	3

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attacked and levulose and trehalose only seldom Groups 18–23, consisting of 14 strains, frequently produced acid from salicin, arabinose, inulin, and raffinose, none of which are attacked by *F meningosepticum* Nine of the 17 strains which reduced nitrates beyond nitrites belonged in these groups

DISCUSSION

Groups 2–17 fulfilled the criteria for *F meningosepticum* They represent 88 strains corresponding to a frequency of 0 3 per cent of the specimens examined The 15 strains in groups 18–23 cannot be classified with certainty, but can be regarded as a more saccharolytic variant of *F meningosepticum*, or as another species of *Flavobacterium* Reduction of nitrates beyond nitrites occurred frequently in these groups

Group 1 is similar to a group designated IIf by *King* (7), who stated that the majority of these strains were isolated from the female genitals

By the isolation of *F meningosepticum* from the female genitals, a possible explanation is provided for the hospital infections caused by that micro organism in the form of meningititis in premature infants The micro-organism was found most frequently in the female urethra but was also found in the rectum

The age distribution of the patients was as could be expected in a venereological material (8), but there was a predominance of females No specific symptoms were found

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Acta path microbiol scand Section B 79, 107–116, 1971

STUDIES ON THE COMPLEMENT FIXATION TEST WITH *MYCOPLASMA PNEUMONIAE* ANTIGEN

6 *Preparation of Cell Antigens with Low Contents of Medium Constituents*

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M pneumoniae cell suspensions prepared by 4 different techniques were examined with regard to the degree of contamination with broth medium constituents, using quantitative protein measurements and gel diffusion tests with antisera against broth medium constituents Unwashed pellets from high-speed centrifugation of broth cultures were found to be highly impure Washing the pellets by repeated resuspensions and centrifugations resulted in an only partial purification, broth medium constituents precipitated as a result of the centrifugation being largely unremovable by this procedure Prefiltration of the broth medium yielded less contaminated, but still impure, pellet antigens Suspensions prepared by scraping off washed layers of *M pneumoniae* grown on a plastic surface did not show gel precipitation reactions of broth medium constituents, their protein figures were relatively high The significance of this finding is discussed The yields from cultures on plastic surfaces were found to be greatly increased by a renewal of the broth medium during the incubation period A filter disc method for harvesting *M pneumoniae* cells from broth cultures is described, the cells being washed upon, and thereafter scraped off from, the surface of Millipore® filter discs type GS (0 22 μ) This method yielded cell antigens of significantly higher purity than the centrifugation method The filter disc method, in combination with prefiltration of the broth medium, is easily practicable for harvesting washed cell suspensions of broth-grown *M pneumoniae* for experimental studies and the production of diagnostic antigens

Suspensions of *M pneumoniae* cells harvested from broth cultures by high speed centrifugation are generally considered to contain varying amounts of broth medium constituents Since the broth medium generally employed (1) is a complex one containing a high proportion of whole horse serum, the medium contaminants in cell suspensions may hamper experimental studies of the properties of *M pneumoniae* cells, and they

Received 22 viii 70

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are highly undesirable in diagnostic antigens used for a specific serological diagnosis of *M pneumoniae* infections The aim of the present work has been to examine the relative merits of four different techniques for processing *M pneumoniae* complement fixing (CF) cell antigens with special regard to the purity of the antigens

MATERIAL AND METHODS

The Bård strain of *M pneumoniae* (3) was cultivated in the PPLO broth and agar media described by *Chanock et al* (1, 2), with some minor modifications as referred to in (3) The production of rabbit antiserum against *M pneumoniae* is described in (3) the serum was used in a dilution containing 4 CF units throughout the present study for linear measurements of the CF titres in the antigenic preparations —The CF microtechnique (modified Takatsy) described by *Sever* (9) was used, employing the Micrometer® system The total test volume was 0 1 ml The fixations were done at +4° C overnight 2½ 50 per cent units of complement and 2 per cent sensitized sheep erythrocytes were used Veronal buffered saline with pH 7 2 was used as diluent —Quantitative protein measurements were made with the Folin Ciocalteu reagent by the method of *Lowry et al* (7), using the technical procedures laid down in (6) Readings of absorption were made at wavelength 500 mμ in a Perkin Elmer Spectrophotometer and the protein figures read from a standard curve based upon diluted normal human serum —Antisera against PPLO broth medium constituents were produced by immunizing one rabbit with whole sterile broth medium and another rabbit with the unwashed pellet from centrifugation of sterile broth medium at 23 000 G for 45 min resuspended in 1/100 vol diluent Both immunizing antigens were first dispersed in an equal volume of Freund s incomplete adjuvant (Bacto) for 2 intramuscular immunizations (each of 2 × 1 ml) thereafter 3 doses (0 3 1 ml) of untreated immunizing antigens were given intravenously The rabbits were bled 14 days after the last i v dose —The gel diffusion method was used for the serological testing of the antigenic preparations with the rabbit antisera against PPLO broth medium constituents The technique was essentially that described by *Ouchterlony* (8) The tests were performed in 0 9 per cent agarose (Seravac) in Tris buffer pH 7 6 upon glass slides Each reservoir was 3 mm in diameter and the distance between the edges of two adjacent reservoirs was 5 mm The slides were incubated at room temperature in a humidified atmosphere up to 5 days Photographs were taken of the unstained precipitation lines in oblique light —Sonications were made in an M S E Ultrasonic disintegrator 60 W at 20 000 c p s

EXPERIMENTAL

Cell suspensions of *M pneumoniae* were prepared by four different techniques

A) (Pellet unwashed) A broth culture was made as described in (3) with finely cut agar culture as the inoculum After about 7 days incubation the agar masses were removed by filtration through sterile gauze The broth culture was then centrifuged at 23 000 G for 45 min and the pellet resuspended in 1/100 vol diluent

B) (Pellet washed × 2) The pellet obtained as described under A was resuspended in 30 vol diluent and centrifuged at 23 000 G for 30 min This procedure was performed twice After the last centrifugation the pellet was resuspended in diluent in 1/100 vol of the original broth culture

C) (Grown on a plastic surface) 100 ml broth medium was poured into a 250 ml bottle made of autoclavable polypropylene and 8 ml of a 4 day-old broth culture freed from agar pieces was added as inoculum (Exps II and III) 25 ml broth medium was poured into a presterile Petri dish with a diameter of 8 4 cm made of polystyrene and 2 ml inoculum broth culture was added The dishes were then sealed airtight (Exps IV, V and VI)

The broth medium was renewed at day 4 the primary medium containing broth grown *M pneumoniae* being removed and discarded and replaced with an equal volume of fresh prewarmed broth medium This procedure markedly increased the density of the macroscopically visible *M pneumoniae* cell layer growing on and adhering to the bottom of the plastic vessel (see Results) After a total of 7 days incubation the broth medium was drained off and the plastic vessel filled up with sterile saline The saline was changed 4 times at intervals of 3–5 min After the last washing the layer of *M pneumoniae* cells attaching firmly to the plastic surface was scraped off in a convenient volume of diluent with a blunt glass pipette The primary suspensions obtained in this manner were pooled and then concentrated by a single centrifugation at 23 000 G for 30 min and resuspension of the pellet in a small volume of diluent

D) (Filter disc eluate) 20 ml broth culture about 7 days old freed from agar pieces was filtered through a Millipore® filter disc type *GS* (stated pore diameter 0 22 μ) with a diameter of 47 mm under reduced pressure by suction When the filtration was just completed the filter disc was washed by passing 30 ml sterile saline through the filter in the same way This washing procedure was performed 4 times After the last washing 2

ml diluent was applied to the surface of the filter disc which was then scraped lightly with a blunt glass pipette The primary materials harvested in this way were pooled and then concentrated by a single centrifugation at 23,000 G for 30 min and resuspension of the pellet in a small volume of diluent

Of each of the four types of cell suspensions, 4–6 different batches were produced, in independent cultivation experiments One batch of each of the four types were matched and their CF-titres measured in the same set-up on the same day In this examination, each suspension was divided into three portions one portion was measured after inactivation at 56° C for 30 min only, one portion was measured after inactivation and then sonication for 20 min and one portion was measured after treatment at 90–95° C (boiling waterbath) for 30 min Each subportion was double-titrated in a 1 2 titration row and a 1 3 titration row and the CF titre of the subportion read as the dilution giving 50 per cent inhibition of haemolysis

The protein contents of the cell suspensions were measured after they had been homogenized by sonication for 3 min

The protein content of a material in the dilution containing one 50 per cent CF unit was calculated by dividing the protein content of the material by its CF titre

Control materials were prepared for each of the four preparation methods described above, by replacing the broth cultures and inocula containing *M pneumoniae* with sterile media, and otherwise proceeding in exactly the same manner unless otherwise stated The protein contents in the control materials were measured

Prefiltration of the complete, sterile broth medium before it was used for cultivation experiments was effected through Seitz asbestos filters type EKS 2 with a diameter of 14 cm The cultivation exps IV–VI were done with prefiltered broth medium whereas in exps I–III the broth medium was used without a prefiltration

Qualitative gel diffusion tests with antisera against broth medium constituents were done with untreated, undiluted cells suspensions Suspensions showing precipitation lines in the qualitative test were then examined quantitatively, using double titrations of the suspensions starting with 1 2 and 1 3 dilutions respectively The gel precipitation (GP) titre of a suspension was read as the highest dilution giving a distinct precipitation line, easily observable by the naked eye in the unstained slides against one or both of the two rabbit antisera employed Undiluted sera were used throughout the gel diffusion tests

RESULTS

The protein contents in the 4 different types of *M pneumoniae* cell CF antigens are shown in Table 1 The values within one and the same experiment (I–VI) are directly comparable by vertical reading of the Table The values from one experiment to another (horizontal reading of Table 1), however, are not strictly comparable because of the normal variations in sensitivity from one CF set-up to another For each cell suspension, the protein value in the dilution containing one 50 per cent CF unit was generally lower in the sonicated and heated subportions than in the subportion inactivated only, in accordance with the CF-titre increasing effect of these physical treatments upon *M pneumoniae* cell suspensions (see "Discussion") For the three types of cell suspensions prepared from *M pneumoniae* grown in broth culture, the same rank of protein content values was found in all experiments "pellet unwashed' having the definite highest protein values, "filter disc eluate" showing the significantly lowest values, and "pellet washed × 2' having intermediate figures 'Grown on a plastic surface" showed protein values below those in "pellet washed × 2' in exps II and III, whereas the reverse ratio was found in exps IV–VI (see 'Discussion") "Filter disc eluate" showed the definitely lowest protein values of all the four antigen types tested

The qualitative gel diffusion test with antisera against broth medium constituents yielded the same result in all the experiments, those from exps V and VI are shown in Fig 1 b The precipitation patterns were well developed after 1 day's incubation and the photographs were taken after 2 days No change in the precipitation pattern was seen after a further incubation up to 5 days "Pellet unwashed" formed multiple, strong precipitation lines "Pellet washed × 2' gave a weaker, but definite positive GP reaction 'Filter disc eluate" and "Grown on a plastic surface" both showed complete absence of precipitation lines against both antisera The

TABLE 1 *Protein Content in* M pneumoniae *Cell Suspensions Prepared by 4 Different Techniques*

Type of antigen	Exp No I	II	III	IV	V	VI
	31 4	47 4	38 3	10 4	12 3	5 6
Pellet unwashed			23 8	6 1	10 3	7 6
		35 5	18 0	6 7	8 8	4 1
	31 3	36 4	14 5	6 5	5 8	4 2
Pellet washed × 2			16 7	5 1	5 3	4 2
		25 0	11 4	3 6	4 7	2 1
		18 1	13 6	7 1	11 8	8 9
Grown on a plastic surface			11 7	7 1	8 7	8 0
		10 4	7 8	4 3	10 2	6 8
			12 5	4 8	2 9	2 0
Filter disc eluate			7 8	2 0	2 9	2 0
			2 6	2 4	2 3	1 5

The figures in the columns give the protein content figures in μg/ml in the dilution of antigen which contains one 50 per cent complement fixing unit, estimated from the complement fixing titres measured in the antigens after inactivation at 56° C for 30 min only (top figure), sonication for 20 min (middle figure), and heating (90–95° C) for 30 min (bottom figure) respectively Exps IV–VI were performed with prefiltered broth medium (see text)

= not performed

TABLE 2 *Titres of Specific Antigen as Measured in the Complement Fixation Test, and of Broth Medium Constituents as Measured in the Gel Precipitation Test in Cell Suspensions of* M pneumoniae *Prepared by 4 Different Techniques*

Type of antigen	Exp No I CF	I GP	II CF	II GP	III CF	III GP	IV CF	IV GP	V CF	V GP	VI CF	VI GP
	128		96		90		450		320		700	
let unwashed		32		24	145	32	768	32	384	24	512	32
			128		192		700		450		950	
	64		55		55		200		256		384	
let washed × 2		4		4	48	3	256	3	280	4	384	4
			80		70		360		320		768	
			80		55		80		110		115	
›wn on a plastic surface				0	64	0	80	0	150	0	128	0
			140		96		130		128		150	
					80		80		128		225	
er disc eluate					128	0	192	0	128	0	225	0
					384		160		160		300	

CF = Complement fixation titre against serum from a rabbit immunized with *M pneumoniae* cells measured after inactivation at 56° C for 30 min only (top figure) sonication for 20 min (middle figure), and heating (90 95° C) for 30 min (bottom figure) respectively

GP = Gel precipitation titre against sera from rabbits immunized with sterile broth medium and pellet from centrifugation of sterile broth medium, measured in the untreated cell suspension

= Not performed

0 = Gel precipitation lines not detectable using undiluted cell suspensions

GP titres with antisera against broth medium constituents resulting from the quantitative gel diffusion tests on Pellet unwashed and Pellet washed × 2 are shown in Table 2 which also presents the specific CF titres of the *M pneumoniae* cell suspensions studied The following conclusions can be drawn from Table 2 1) The specific CF titres in Pellet washed × 2 are lower than those in Pellet unwashed in all the experiments reflecting a loss of cells during the washing procedure 2) The reduction in GP titres of broth me



Fig 1 a Gel precipitation system for demonstrating constituents of PPLO broth medium Upper part Central well serum from a rabbit immunized with the unwashed pellet from centrifugation of sterile PPLO broth medium Wells at 3 o clock and 9 o clock serum from a rabbit immunized with sterile uncentrifuged PPLO broth medium Wells at 1 and 11 unwashed pellet from centrifugation of sterile PPLO broth medium Wells at 5 and 7 sterile uncentrifuged PPLO broth medium Lower part (control system) the reagents have been distributed to the same wells as in the upper part except that the rabbit hyperimmune sera have been replaced by pre immunization sera from the same rabbits



Fig 1 b Application of the gel precipitation system shown in Fig 1 a to the detection of broth medium constituents in cell suspensions of *M pneumoniae* prepared by 4 different techniques The rabbit hyperimmune sera have been added to the same wells as in Fig 1 a upper part Well at 11 o clock Pellet unwashed Well at 1 Pellet washed × 2 Well at 5 Filter disc eluate Well at 7 Grown on a plastic surface Upper part exp V Lower part exp VI

dium constituents which results from the washing procedure of pellet antigens is significantly greater than the reduction in specific CF titres indicating a purifying effect of the washing procedure 3) By comparing the specific CF titres of the four types of cell antigen preparations and the GP titres of broth medium constituents in the pellet preparations it is easily seen that all the Pellet unwashed and Pellet washed × 2 preparations exhibit GP reactions of broth medium constituents when diluted to or being at the same level of specific CF activity as the undiluted Grown on a plastic surface and Filter disc eluate preparations none of which showed this kind of reaction

Protein Measurements in Control Preparations

A Scrapings from polypropylene bottles 2 exps 1) 120 μg/ml, 2) not detectable (n d)

B Scrapings from polystyrene Petri dishes, 4 exps , all n d

C Filter disc eluate, 1 exp without prefiltration of the broth medium 20 μg/ml 3 exps with prefiltered broth medium 1) n d , 2) 15 μg/ml, 3) n d The control preparations A–C were tested in the native state without a final centrifugation

D The protein contents in 4 different batches of whole PPLO broth medium were measured to an average of 18,400 μg/ml, minimum value 17,000, maximum value 19,400 μg/ml

E The protein contents in pellets from sterile broth medium centrifuged at 23,000 G for 45 min, resuspended in 1/100 vol diluent are given in Table 3, which will be referred to in more detail in the following chapter

Effect of Prefiltration of the Broth Medium

The native PPLO broth medium is moderately opaque, some sediment being formed upon standing The prefiltration procedure yielded crystal clear broth media Indications of the effect of prefiltration upon the protein content of the antigens can be drawn from Table 1 Although figures based upon CF-titres measured in different set ups are not directly comparable a comparison of average values for the exps with and without prefiltration seems justifiable The ratios between the average protein figures (on a one 50 per cent CF unit dilution level) of the antigens prepared without a prefiltration, and those made up using prefiltered broth medium, are given below in the following order for the three subportions of each antigen type inactivated only/sonicated/heated

Pellet unwashed 4 3 1/3 0 1/4 1 1
Pellet washed × 2 5 0 1/3 4 1/5 2 1
Grown on a plastic surface 1 7 1/1 5 1/1 3 1
Filter disc eluate 3 9 1/3 4 1/1 2 1

It is concluded that prefiltration of the broth medium has a reducing effect on the protein content of pellet antigens, both unwashed ones and those washed × 2, whereas no such effect can be concluded from the figures for antigens prepared from *M pneumoniae* grown on a plastic surface The data for filter disc eluates using broth medium without a prefiltration are insufficient, but the total data regarding this type of antigen are compatible with the assumption that the prefiltration procedure may have a lowering effect upon the protein content of antigens prepared from filter disc eluates —Sterile broth medium formed distinct pellets following centrifugation at 23 000 G for 45 min this was also true when a prefiltration was applied indicating that a precipitation of broth medium constituents occurred as a result of the centrifugation procedure Table 3 shows that the control pellets from prefiltered broth medium contained less protein

TABLE 3 *Protein Contents in μg/ml in Unwashed and Washed Pellets from Sterile PPLO Broth Medium Centrifuged at 23 000 G for 45 min and Resuspended in 1/100 Vol*

Prefiltration of the PPLO broth medium performed	No			Yes		
Exp No	I	II	III	IV	V	VI
Pellet unwashed	2800	3750	3100	2250	1575	2000
Pellet washed × 2	1000	1100	600	50	115	200

than the pellets from sterile broth medium centrifuged without a prefiltration. The Table also shows that a higher proportion of the protein in the unwashed control pellets was removed by the washing procedure in the exps. using prefiltered broth medium.

Anticomplementary effect (AC) was found in all the four different types of *M. pneumoniae* cell suspensions when measured in diluent, the difference between the specific CF titre and the AC titre being 2–4 log. dil. steps in most of the experiments. Heating to 90–95° C for 30 min either greatly reduced the AC effect or removed it completely in all four types of antigens.

Special Findings Regarding the Technique of Cultivating M. pneumoniae *on a Plastic Surface*

The introductory experiments with this technique gave variable and often poor yields of cells. Three pilot exps. were thereafter designed to evaluate the effect of a renewal of the medium during the incubation period, the technique of which is described under Experimental. The procedure resulted in a significant increase of the density of the cell layer attaching firmly to the plastic surface after the last washing, easily seen macroscopically. The cell layers were scraped off in equal volumes of diluent and the CF-titres measured directly in the resulting cell suspensions without a centrifugation, the results being presented in Table 4.—Three different plastic qualities were thereafter tested.

Fig. 2. Colonies of *M. pneumoniae* growing on a polystyrene surface. Magnification × 125.

TABLE 4. *Effect of a Renewal of the Broth Medium During the Incubation Period on the Growth of* M. pneumoniae *on a Plastic Surface in 3 Experiments*

Exp. No.	Type of plastic vessel	Renewal of the broth medium at day 4	CF titre of adherent cell layer washed × 5 at day 7
1	Polystyrene Petri dishes	No Yes	1 6
2	Autoclavable polypropylene bottles	No Yes	1 16
3	Autoclavable polypropylene bottles	No Yes	2 48

1) Autoclavable polypropylene (see "Experimental")

2) Polystyrene (see "Experimental")

3) Autoclavable polycarbonate (250 ml bottles)

All the three types of plastic vessels were found satisfactory for cultivating *M pneumoniae* on a plastic surface, the presterile, disposable polystyrene Petri dishes being considered the most practical in use.—Microscopic examination of *M pneumoniae* growing on a plastic surface showed that the growth pattern was that of colony formation (Fig 2)

DISCUSSION

"Pellet unwashed" has been prepared according to the original principle for the production of *M pneumoniae* CF cell antigen described by *Chanock et al* (1)

"Pellet washed × 2" has been prepared using the washing technique consisting of repeated centrifugations and resuspensions of the cells, which have been widely employed in different laboratories as a routine method for purifying *M pneumoniae* pellet cell suspensions

"Grown on a plastic surface" was developed in analogy to the technique of cultivating *M pneumoniae* on a glass surface as described by *Somerson et al* (11) Attempts by the present author to cultivate *M pneumoniae* on an untreated Pyrex® glass surface (other glass qualities not tested) were unsuccessful, the cell layer adhered only loosely to the glass surface and was readily detached by the first washings *Taylor-Robinson et al* (12) reported the adherence of mycoplasmas to plastic surfaces, the potentialities of this phenomenon in the production of washed cell suspensions of *M pneumoniae* have been examined in the present work Using a renewal of the broth medium during the incubation period as earlier described, the method was found an easy and practicable one for production of great amounts of *M pneumoniae* cell suspensions

"Filter disc eluate" is based upon the earlier findings by the present author regarding the efficiency of Millipore® filter discs type GS (0 22 μ) in retaining *M pneumoniae* colony forming units from broth cultures, and the effect of the washing procedure of the cell bearing filter discs in freeing the cells from not cell bound CF antigen (5) In the present work, the value of the same technique in freeing broth grown *M pneumoniae* cells from medium constituents has been investigated

Since the PPLO broth medium is especially rich in proteins, the protein figures of the antigens are considered a valuable parameter of the degree of contamination with broth medium constituents As the aggregation phenomenon in cell suspensions of *M pneumoniae* will have an influence on their CF-titres (4), but not on their protein values, subportions of each cell suspension were subjected to treatment with 2 different physical methods (sonication, heating) known to increase their CF-titres, probably through a dispersing effect (4) The sonication time (20 min) was based upon the findings regarding the effect of sonication at different intervals upon the CF-titres of unwashed pellet antigens of *M pneumoniae* described in (4) Table 2 confirms that the CF-titres in the sonicated and heated subportions are generally higher than those found in the subportions inactivated only Table 1 shows that the same general results are found regarding the relative protein figures of the four types of cell suspensions, regardless of which of the three subportion values of the antigens is chosen for comparison

The following conclusions are drawn concerning the purity of the four antigen types

'Pellet unwashed' is heavily contaminated with broth medium constituents as judged from both of the two parameters employed in the present study This antigen is considered to contain three types of contamination 1 whole broth medium soaking the pellet 2 broth medium constituents in solution in the uncentrifuged medium and precipitated as a result of the centrifugation, 3

(when prefiltration is not used) broth medium constituents not in solution in the uncentrifuged broth medium

"Pellet washed × 2" has a lower content of medium constituents than "Pellet unwashed", reflecting that the washing procedure of repeated centrifugation has some purifying effect. This conclusion is drawn from the lower protein values (Table 1) and the weaker qualitative GP reactions of broth medium constituents (Fig 1b) in "Pellet washed × 2", and from the fact that the washing procedure results in a greater reduction of the GP titres of broth medium constituents than of the specific CF titres (Table 2). However "Pellet washed × 2" is also a significantly contaminated type of antigen, broth medium constituents were readily demonstrated in all the preparations of this type by means of the GP tests, and their protein values were clearly higher than those of the "Filter disc eluates". It seems reasonable to assume that whole broth medium soaking the unwashed pellet is removed by the washing procedure of repeated centrifugations whereas the broth medium constituents precipitated as a result of centrifugation are largely unremovable by this procedure the total result being an only partial purification

"Grown on a plastic surface" showed lower protein figures than did "Pellet washed × 2" in the exps performed without a prefiltration of the broth medium (II, III) whereas the contrary was the case when prefiltered broth medium was used (exps IV–VI). This is probably explained by the protein lowering effect of the prefiltration procedure upon the pellet antigens. The relatively high protein figures of "Grown on a plastic surface" in the absence of GP reactions of broth medium constituents cannot be definitely explained by the results of the present experiments. The morphology of *M pneumoniae* cells growing in colonies on a plastic surface may, however be different from that of cells growing in broth culture. The CF antigen activity of *M pneumoniae* is known to be associated with lipids forming part of the outer cell membrane (10). Variations in growth pattern affecting the membrane/cell content—ratio must thus be expected to influence the amount of mycoplasma protein on a fixed level of CF activity in *M pneumoniae* cell suspensions. The protein figure, considered to be a valid parameter of the relative degree of purity of the three antigen types which were all harvested from broth grown *M pneumoniae*, may thus be less valuable in the comparison of cell suspensions resulting from different cultivation techniques. More research seems desirable as regards the technique of cultivating *M pneumoniae* on a plastic surface including methods for differentiating between medium proteins and mycoplasma proteins before a final judgment is made regarding the degree of purity of this type of *M pneumoniae* cell suspensions

"Filter disc eluate" exhibits the highest degree of purity of the three antigen types made up from broth-grown *M pneumoniae* studied as appraised from both the protein figures and the results of the GP tests. The filter disc method must be considered lenient towards the mycoplasma cells. By using a filter equipment with a diameter greater than that employed in the present work the method is easily practicable for harvesting washed cell suspensions of broth grown *M pneumoniae* for experimental studies and the production of diagnostic antigens

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Acta path microbiol scand Section B 79, 117-122, 1971

PROTEOLYTIC ENZYMES AND BIOLOGICAL INHIBITORS

VI *Antibodies in Animal Sera against the Proteinase of* Corynebacterium pyogenes

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Rabbits and pigs artificially infected with washed cells of *Corynebacterium pyogenes* were shown to produce inhibitory antibodies against the bacterial proteinase, as tested by electrophoresis of the sera, combined with the casein precipitating inhibition test (electrophoretic CPI test) The antibodies were usually detectable about 14 days after inoculation Sera of animals not inoculated with microorganisms were also examined In sera collected from 10 randomly selected animals of each of the species man, horse, chicken, rabbit and dog antibodies against *Corynebacterium pyogenes* proteinase could not be demonstrated On the other hand, six, four, and two sera out of ten from cattle, pig and sheep respectively, were found to contain such antiproteinases In the sera from an additional 25 selected pigs, antibodies were demonstrated in 16, most of these pigs were shown to have chronic corynebacterial infection Antibodies against *Corynebacterium pyogenes* proteinase were demonstrated in most of the sera from 34 selected cattle of different ages, especially from the older ones In some of these animals the bacterium was isolated from pyogenic processes, in other animals there were strong indications of a corynebacterial infection, but in other animals there was no direct indication of such an infection although previously experienced infections, with or without clinical manifestations are also supposed to have occurred in these cases The diagnostic value of qualitative or quantitative determination of such antiproteinases in cattle and pig is considered to be of limited value due to the frequent occurrence of such antibodies

Many proteolytic enzymes are shown to have good antigenic properties when inoculated, with adjuvants, into rabbits, either as crude enzyme containing materials, or as highly purified products (*Sandvik* 1962, *Fossum*, in press d) By electrophoresis of the sera of immunized animals, combined with the casein precipitating inhibition test (electrophoretic CPI test), the activity of the antienzymes can be distinguished from that due to the naturally occurring inhibitors (*Sandvik* 1962, *Fossum*, in press a, d)

After certain bacterial infections antibodies against some extracellular enzymes produced by the causative bacteria, can be demonstrated in the sera of the patients This is especially the case after certain strepto-

Received 30 viii 70

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coccal infections in man, where increased inhibitory activities against streptolysin O, hyaluronidase, streptokinase, diphosphopyridinenucleotidase, and desoxyribonuclease have been demonstrated (*Bernhard* & *Stollerman* 1959, *McCarthy* 1949). Quantitative determination of such antibodies is sometimes of diagnostic value (*Christie* 1969). The presence of antibodies against proteolytic enzymes after an infection due to a proteinase producing microorganism does not seem to have been reported previously, although *Todd* (1947) reported some investigations along these lines in horse and human sera as to inhibitory activity against streptococcal proteinases.

During investigations of the naturally occurring inhibitors in animal sera (*Fossum*, in press a), it was found that the sera from some animals, especially cattle and pig, contained inhibitors against *Corynebacterium pyogenes* proteinase in the γ globulin region of the sera subjected to electrophoresis.

The aim of the present work was to investigate the possible production of humoral antibodies against *Corynebacterium pyogenes* proteinase after artificial infection with this microorganism. The presence of inhibitors against the proteinase in the γ globulins in sera from randomly collected individuals and from animals with or without a known history of infection was also investigated.

MATERIALS AND METHODS

Artificial Infection of Animals with Corynebacterium pyogenes

Rabbits and pigs were used for artificial infection with *Corynebacterium pyogenes*. The strain used had been isolated from an abscess in the spinal column of a pig with tail sores. The organism was cultivated in nutrient broth for two days and the cells were washed three times in saline before inoculation. Two rabbits were inoculated intravenously with approximately 10^7 cells and two other rabbits received the same number of cells intraperitoneally in a diffusion chamber (Millipore[1]), using filters with a pore size of 0.22 μ. Four pigs, about ten weeks old, were inoculated in the tails about 3 cm from the tip with approximately 10^7 cells after previous emaceration of the inoculation site. Blood samples taken immediately before inoculation and later at 2–6 days intervals were examined for the presence of antienzymes.

Sera from Other Animals

Initially ten individual sera from each of the eight different animal species were examined for the presence of inhibitory substances against *Corynebacterium pyogenes* proteinase in the γ globulins. The samples of cattle, pig, sheep, horse and hen were taken from randomly selected slaughter animals. No clinical or postmortal examinations were performed on these animals. Blood samples from rabbits were taken from healthy laboratory animals, samples from dog were obtained partly from healthy, partly from diseased animals, and human blood was taken from healthy adult individuals. Additional blood samples were taken from 25 selected pigs and 34 selected cattle that either were hospitalized or were to be slaughtered. These animals were usually subjected to clinical and/or postmortal examination, and in some cases bacteriological examinations were also performed.

Determination of Antienzymes

Separation of the naturally occurring inhibitors and the antienzymes, and the demonstration of the inhibitory activity of the antienzymes, were performed by electrophoresis of the sera combined with the casein precipitating inhibition test (electrophoretic CPI test) (*Fossum*, in press a, b, c, d). The *Corynebacterium pyogenes* proteinase used was obtained by cultivating the bacterium (*Corynebacterium pyogenes* NVH* 431) in sterilized skim milk medium for one week at room temperature and subsequent precipitation of the supernatant by 80 per cent saturation with ammonium sulphate. After dialysis of the water soluble precipitate against distilled water, the crude enzyme-containing material was, in an appropriate dilution, used for developing. The other proteolytic enzymes used (proteinases from *Aspergillus oryzae*, *Bacillus cereus*, *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), were prepared as described previously (*Fossum*, in press a).

RESULTS

Prior to inoculation no inhibitory activity against the proteinase of *Corynebacterium pyogenes* could be detected in the γ globulins

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[1] Millipore Corporation, Bedford, Mass., U.S.A.

* NVH: The Culture Collection of the Department of Microbiology and Immunology, Veterinary College of Norway.

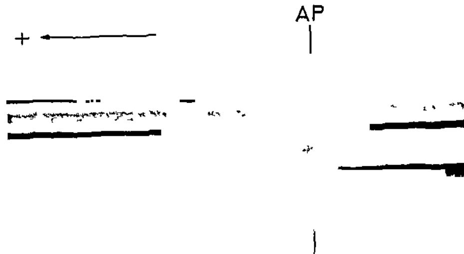


Fig 1 Electrophoretic CPI test of serum from pig inoculated with *Corynebacterium pyogenes* The enzymes used are (downwards) Proteinases from *Corynebacterium pyogenes* and *Bacillus cereus*, and swine trypsin (0 005 mg per ml) The electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 hours The zone of inhibition near the line of application (AP) is due to the induced antienzyme against *Corynebacterium pyogenes* proteinase, while the zones of inhibition on the anode side are due to the naturally occurring inhibitors in pig serum

of the rabbits and pigs used From one of the two rabbits inoculated intravenously, a trace of inhibitory activity in the γ globulin region of the electrophorized serum could be demonstrated on the 10th day after infection The rabbit was killed on this day because of lameness in the hind quarters due to an abscess in the spinal column Specific inhibitors were demonstrated on the 14th day after inoculation for the other rabbit One month after infection, the inhibitory activity in the γ globulins could be demonstrated by the electrophoretic CPI test in serum dilutions up to 1 50 Specific inhibitors were first demonstrated 28 days after infection in the two rabbits which received the bacteria intraperitoneally in a diffusion chamber The induced inhibitory activity increased during the next 14 days Upon autopsy it was found that the diffusion chambers were incapsulated, and abscesses from which *Corynebacterium pyogenes* was isolated, were localized in the connective tissue

In the four inoculated pigs induced inhibitors against *Corynebacterium pyogenes* proteinase were first demonstrated 14–20 days after inoculation after which the inhibitory activity increased rapidly during the next 10 to 14 days Fig 1 shows the zones of inhibition caused by the serum of one of these pigs 30 days after inoculation A marked zone of inhibition against the *Corynebacterium pyogenes* proteinase is seen around the line of application Prior to inoculation this type of inhibition was not observed The other proteolytic enzymes tested were not inhibited in this area Upon autopsy of the pigs 40 days after inoculation, abscesses were found in all cases at the inoculation sites as well as in the spinal columns Abscesses were also found in the lung for one of the pigs *Corynebacterium pyogenes* was isolated in all cases, in pure cultures, from the abscesses

Inhibitors in the γ globulin area of the electrophorized sera against the *Corynebacterium pyogenes* proteinase could not be demonstrated in any of the randomly collected sera investigated from man, horse, chicken, healthy rabbits, or dog On the other hand, such inhibitors were demonstrated in six of the ten bovine sera, four of the pig sera and two of the sheep sera

The results obtained by examination of 25

TABLE 1 *The Occurrence of Antibodies against* Corynebacterium pyogenes *Proteinase in Pig Sera in Relation to Clinical (C) or Autopsy (A), and Bacteriological Findings*

Number of pigs	Clinical or autopsy findings	Bacteriological findings	Specific inhibitors against *Corynebacterium pyogenes* proteinase
4	No findings (A)	Not investigated	—*
1	No findings (A)	Not investigated	+ + + +†
2	Pneumonia (A)	—	—
10	Tail sores of older date with suppurations with or without abscesses in other organs (A)	+§	+ + + +
3	Broken tails or tail sores without suppurations No abscesses in other organs (A)	—	—
2	Tails sores of newer date Hemorrhagic infarcts in the lungs (Acute or subacute) (A)	+	+ +
3	Arthritis (chronic) (C)	+	+ + + +

* — *Corynebacterium pyogenes* not isolated or antienzymes not observed
† + + + + + + Varying amounts of antienzymes observed
§ + *Corynebacterium pyogenes* isolated

selected pigs for the presence of specific inhibitors against *Corynebacterium pyogenes* proteinase are shown in Table 1 The autopsy or clinical findings and the results of the bacteriological investigations are also presented It can be seen that in all cases when *Corynebacterium pyogenes* was isolated specific inhibitors against its proteinase were also present in the animal sera Specific inhibitors were not observed in sera from four of the five healthy pigs or in the pigs with tail lesions but without any manifest corynebacterial infection Large amounts of antienzymes were demonstrated in sera from one pig with no sign of previous corynebacterial infection Pure cultures of *Pasteurella multocida* were isolated from the lungs of the two pigs with pneumonia included in the table

Table 2 presents the occurrence of specific inhibitors against *Corynebacterium pyogenes* proteinase in sera from 34 cattle in relation to the clinical and in a few cases autopsy and bacteriological findings It can be seen that these inhibitors which in all cases were localized at, or near the line of application were frequently present in cattle sera especially in older animals By electrophoresis of diluted sera the activity of these inhibitors was demonstrated in some cases after a dilution of 1 100 while the effect of the naturally occurring inhibitors upon *Corynebacterium pyogenes* proteinase could not be observed in dilutions higher than 1 40–1 50 under the same conditions

In the case of the other microbial enzymes tested inhibitory activity in the γ globulin area was occasionally observed against proteinases of *Bacillus subtilis* and *Aspergillus oryzae* This activity was weak and was not found to correlate with the inhibitors against *Corynebacterium pyogenes* proteinase

DISCUSSION

The present experiments with inoculation of washed cells of *Corynebacterium pyogenes* into rabbits and pigs indicate that this bacterium in the host organism produces sufficient amounts of the extracellular proteinase to provoke an immunological response which

TABLE 2 *The Occurrence of Antibodies against* Corynebacterium pyogenes *Proteinase in Cattle Sera in Relation to the Age of the Animals, and the Clinical (C) or Autopsy (A), and Bacteriological Findings*

Number of animals	Age (approximate)	Clinical or autopsy findings§	Bacteriological findings	Specific inhibitors against *Corynebacterium pyogenes* proteinase
3	<2 months	No findings (C)	Not investigated	—*
1	1½ month	Polyarthritis (C)	+	+ +
1	2 months	Abscess (subcutaneous) (C)	+	+ +
1	1 year	Botulism (C)	Not investigated	—
3	5 months	Coughing Lung worm invasion (C)	—	+ +
1	5 years	Pneumonia (A)	+	+ + + +
2	4–5 years	Traumatic indigestion (A)	+	+ + + +
3	3–5 years	Traumatic indigestion (C)	Not investigated	+ + + +
8	2–6 years	Chronic indigestion (C)	Not investigated	+ + + +
1	4 years	Ketosis (C)	Not investigated	+ + + +
1	4 years	Botulism (C)	Not investigated	+ +
1	6 years	No findings (C)	Not investigated	—
8	1 5 years	Different diagnosis not supposed to be related to *Corynebacterium pyogenes* infection (C)	Not investigated	+ +

§ The clinical investigations were performed by veterinary surgeons at the Department of Internal Medicine, The Veterinary College of Norway and the data obtained from veterinary surgeon H E Utklev who also supplied most of the sera

* The same symbols used as in Table 1

results in production of specific antibodies As far as rabbits are concerned, this antibody formation seems to be similar to that caused by immunization with crude, or purified, proteolytic enzymes mixed with adjuvant (*Sandvik* 1962, *Fossum*, in press d) The purpose of introducing the organism enclosed in diffusion chambers was to investigate if antibodies against the bacterial extracellular proteinase could also be produced when the organism was maintained inside a chamber which allowed the passage of nutrients and bacterial products However, the pericapsular abscesses showed that reproductive elements of the bacterium had leaked out Whether this was due to inadequate constructing, or filling, of the diffusion chambers or to a passage of the bacterium itself, or of smaller reproductive elements of the organism through the filter pores (*Schulz & Gerhardt* 1969), has not yet been elucidated

Artificially provoked infection with *Corynebacterium pyogenes* in pigs also induced the production of specific proteinase inhibitors in a similar manner as in rabbits The inhibitors are therefore considered to be normal antibodies In the spontaneous cases of subacute or chronic forms of corynebacterial infection in pig, the specific inhibitors against *Corynebacterium pyogenes* proteinase are also supposed to be due to such antibodies (Table 1) Although high amounts of specific inhibitors were present in the one case in which no clinical or autopsy findings were made, the result may be due to a previously experienced, and healed, corynebacterial infection

In cattle, the occurrence of inhibitors against *Corynebacterium pyogenes* proteinase in the serum γ globulins was, in the present material, surprisingly high, especially in older individuals This is so even when one

considers the high frequency of *Corynebacterium pyogenes* infections in this animal species. However, the mode of selection of the material must be considered in this connection. In some of these cases a corynebacterial infection was shown to exist, in some others it very probably existed, or had existed. In still other cases there was no indication of such an infection. In the latter cases the inhibitory activity in the γ globulin region is also considered to be due to specific antienzymes produced as a result of a previously experienced infection with, or without, clinical manifestations, or to a latent or not diagnoseable infection. The lower incidence of the antibodies in younger animals supports this theory, as does the absence of these inhibitors in animals not usually affected by *Corynebacterium pyogenes*.

The use of the qualitative or quantitative demonstration of the said antiproteinases for diagnostic purposes, for example, in cases of chronic indigestions in cattle, or pyobacillosis in pig, seems to be of limited value, because of the presumed high frequency of light, or latent, infections with *Corynebacterium pyogenes* in these animal species.

The author is grateful to veterinary surgeon *H. E. Utklev* at that time working at the Department of Internal Medicine, The Veterinary College of Norway, for supplying most of the cattle sera, and for providing the relevant information from the clinical and autopsy findings on the animals.

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Acta path microbiol scand Section B 79, 123-124, 1971

BRIEF REPORTS

RESPIRATORY INFECTION WITH PARAINFLUENZA 1, SENDAI VIRUS IN GNOTOBIOTIC AND CONVENTIONAL MICE

Miklos Degré and *Tore Midtvedt*

It has been established both clinically and by experimental studies that viral infection of the respiratory tract may be followed by secondary bacterial invasion (2, 5) While some of the secondary invaders are recognized as pathogenic species the majority are derived from the normal resident flora of the upper respiratory tract The pathogenicity of the latter organisms remains poorly defined The contribution of these bacteria to the infectious process has not been clearly delineated Recent studies in our laboratory indicate that the secondary invaders of low pathogenicity may participate in the respiratory infection of mice (3) The continuous administration of Penicillin G to mice infected with parainfluenza 1, Sendai virus reduced the mortality and the polymorphonuclear (PMN) leukocyte accumulation in the lungs (1) The most likely explanation for the effect of penicillin was an elimination of the spontaneous bacterial invasion which follows viral inoculation This report extends these studies to germfree (GF) mice in order to define the role of resident flora by ensuring a complete control of the absence of it

Young albino mice of CD 1 strain were used One group 15 mice was obtained GF from the Charles River Mouse Farms, Wilmington Mass USA and was kept under germfree conditions as described elsewhere (6) Negative cultures at the termination of the experiment proved that the mice remained bacteria free Groups 2 and 3 included conventionalized mice of the same strain All three groups were inoculated with 10^4 EID_{50} parainfluenza 1 virus Sendai strain, as previously described (2 6) The GF group and one of the conventionalized groups were given water to drink the third group water containing Penicillin G 8000 IU per ml With the average daily water consumption of 5–6 ml, the mice were administered roughly 4 million IU per kg body weight The serum level of Penicillin was about 1 5 µg per ml determined by tube dilution method All three groups were observed for 10 days and mortality was recorded daily At the end of the observation period the survivers were sacrificed, and their lungs were examined for presence of bacteria and for white cell count, by the methods previously described (1) The results are summarised in Table 1 It seems to be clear that the outcome of disease is correlated with the chance of bacterial contamination A higher proportion of mice died, and they died earlier in the conventional untreated group where bacterial spreading occurs freely, than in the treated group and still less in the GF group It appears that parainfluenza 1 virus alone produces a mild infection, even though the virus seems to multiply in the respiratory organs of GF mice Significant titers were demonstrated in the lung wash outs, 10 days after inoculation, by the hemadsorption method using Vero cells and 0 05 per cent of guinea pig erythrocytes The lower susceptibility of GF mice to viral infection is not a general phenomenon (7 8 9) Therefore no conclusion of general validity can be drawn from the present experiment

The extent of PMN leukocyte accumulation seems to be correlated with the presence of bacteria (1 2) The differences in the present experiment are not significant although there is a clear tendency to obtain increasing proportion of PMN leukocytes with increasing chance of bacterial contamination

The present results support the concept, that if the defense mechanisms are weakened one way or another the resident bacterial flora invades the lower parts of respiratory tract and in some cases produces disease The defense mechanisms might be reduced by f x immunosuppressive drugs (9) or as in the present study by virus infection

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Received 20 xi 70 *from Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, Universitetet of Oslo Rikshospitalet, Oslo Norway*

TABLE 1 *Mortality, White Cell Accumulation and Presence of Bacteria in the Lungs of Germfree and Conventional Mice following Inoculation with Parainfluenza 1 Virus*

	Mortality		Survival		White cells in the lung			Bact in t lun
	Total	Per cent	Total days	Mean	Total	PMN per cent	Macrophages per cent	
Conventional mice								
No treatment	12/15	79*	117	7 80*	ND	21†	12	3/3
Penicillin G treatment	7/15	46*	137	9 13*	ND	18†	18	1/8
Germfree mice	1/15	7*	147	9 70*	8×10^4	13†	42	0/1

* $p < 0\,05$
† $p > 0\,05$
§ Survivers after 10 days observation
ND Not done

Summary

The presence of resident bacterial flora in the respiratory tract influences the outcome of infectious disease following parainfluenza 1 virus infection, and, to some extent also the leukocyte invasion in the lungs

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ELIMINATION OF INTERCURRENT DEATH AMONG RABBITS INOCULATED WITH *TREPONEMA PALLIDUM*

H J Skovgaard Jensen

A high incidence of early death among rabbits after inoculation with *Treponema pallidum* (Nichols' pathogenic strain) is observed in TPI laboratories in Denmark (*Jorgensen* 1968) Norway (*Erichsen* 1968), and Sweden (*Gudjónsson* & *Skog* 1968, 1970) This phenomenon seems to have been recognized first in Denmark and later in Norway and Sweden, but it has not been reported from other countries

In Denmark Nichols strain of *T pallidum* (*Nichols* & *Hough* 1913) has been maintained in rabbits since 1951 These treponemes were received,

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respectively, from Johns Hopkins Hospital, Baltimore, in April 1951, and from University of California, Los Angeles, in January 1966 The two substrains are propagated by intratesticular inoculation of suspensions of treponemes obtained from testes every 6th to 10th day, and the rabbits used are all of the same random bred strain During the period 1951 to 1961, the incidence of intercurrent death was less than 2 per cent From 1962 to 1966 the annual incidence rose from 3 to 16 7 per cent (*Jorgensen* 1968) and since then this percentage has gradually increased to 34 7 per cent in 1969

A prominent clinical sign of the intercurrent rabbit disease is fever, starting about the 3rd day after inoculation A varying and apparently unpredictable proportion of the animals then die suddenly, most often on the 4th day according to *Jorgensen* (1968), but death may occur from the 3rd to the 14th day according to *Gudjónsson* & *Skog* (1970) The *post mortem* picture is characterized by serous effusions in the pleural cavities, marked lung oedema and dilatation of the right heart Histologically, the animals do not show any outstanding changes The characteristic gross changes do not appear to occur in other known diseases of the rabbit

So far the aetiology of this intercurrent death of rabbits remains obscure *Jorgensen* (1968) suggested that death might be caused by chemical substances or microorganisms other than the treponemes *Gudjónsson* & *Skog* (1970) concluded from various experiments that the treponemes could not be the sole cause and that their observations did not fit in with an allergic reaction as the cause of fever and death of rabbits

Authors Experiments

Further studies in Denmark have been concentrated on the aetiology of the syndrome and the prevention of intercurrent death Based on the supposition that an unknown agent, together with the treponemes, was being passed from rabbit to rabbit, the following method has so far proved successful in preventing the death of rabbits used for passage of treponemes

On April 21st, 1970, a sample of routinely used suspension of treponemes (Baltimore substrain) from rabbit testes was received from the Department of Treponematoses, Statens Seruminstitut, Copenhagen The sample contained approximately 5 mill treponemes per ml Each of two 8–10 week-old male hamsters was inoculated intradermally in the groins with 0 2 ml of this suspension according to the method described by *Hollander* & *Turner* (1957) None of the hamsters showed any clinical signs of disease following inoculation After sacrifice on the 24th post inoculation day, a suspension of treponemes in physiological saline was prepared from the moderately enlarged inguinal lymph nodes and transferred to new male hamsters In this way the Baltimore substrain had a total of five hamster passages at intervals of 24 to 31 days On September 3rd, treponemes from the fifth hamster passage were transferred back intratesticulary to four rabbits of the same rabbit strain as that used by the Department of Treponematoses When signs of orchitis were present, the treponemes were transferred to four new rabbits In this way the Baltimore substrain had 10 consecutive passages in rabbits until December 28th, 1970 Similarly, the Los Angeles substrain of the Nichols' strain was transferred to hamsters (and simultaneously to 8 rabbits, two of which died on day 4 after inoculation) on September 14th, but this strain was transferred back to rabbits already after one passage, *i e* after 28 days Up to December 22nd this strain has had five passages in rabbits

All 60 rabbits that received treponemes originating from the hamsters were observed for fever and other signs of disease until passage to new rabbits or to the 14th post inoculation day None of the animals died Five of the 40 rabbits inoculated with the Baltimore substrain showed fever for a single day, three of these were used for new passages in preference to rabbits which did not show fever One of the 20 rabbits inoculated with the Los Angeles substrain showed fever for one day and was also used for a new passage

From the initiation of the first hamster passage and until December 28th, 1970 the Department of Treponematoses has continued their routine passages of treponemes in rabbits A total of 998 rabbits were inoculated during this period and 393 *i e* 39 4 per cent, of these animals died

It is concluded that passage of the two *T pallidum* substrains through hamsters has removed the agent responsible for the pleural effusion disease of the rabbit. The question whether this intercurrent disease will reappear in continued passages cannot be answered as yet However, if this should happen, treponeme infected testes from our passages of the two substrains are now preserved in liquid nitrogen to be used for the start of new passages in rabbits

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Acta path microbiol scand Section B 79, 126–128, 1971

INDUCTION OF ANTIBODY SYNTHESIS AGAINST SULPHANILIC ACID IN RABBITS

Helper Function of New Antigenic Determinants Introduced in Autologous Protein

B Rubin and *B Aasted*

It has been suggested that more than one antigenic determinant is required for immunogenicity (*Rajewsky et al* 1969) This statement is based mainly on the following conclusion The secondary antibody response to a given hapten is poor if the carrier molecules used for primary and secondary stimulation are different (*Katz et al* 1970, *Mitchison* 1970), or if the experimental animals are rendered tolerant to the carrier molecule (*Green et al* 1968)

Accordingly, it may be postulated that an immune response to a given hapten coupled to an autologous protein molecule is due to the introduction of new antigenic structures into the carrier molecule during the coupling reaction

In order to test this hypothesis, rabbits were stimulated with sulphanilic acid coupled to RSA and BGG[1] The experiments showed that new antigenic structures are introduced into the carrier molecule during the coupling process, and that the immune response to these new antigenic determinants follows the normal pattern of an immune response to heterologous carrier determinants

Materials and Methods

Twelve week old random bred rabbits (females) were supplied by the animal farm of Statens Seruminstitut RSA and BGG were used as carrier molecules Sulphanilic acid was coupled to N chloroacetyltyrosine and proteins by diazotation The number of Sulph groups[1] bound per mole protein was determined in accordance with *Tabachnick & Sobotka* (1950) The hapten-carrier complexes used were as follows $Sulph_{11}RSA$ and $Sulph_{10}BGG$ The double diffusion method (*Rubin* 1970) was used to determine the serological specificity of the antigens and antisera

Hapten specific passive haemagglutination was carried out according to Ingraham (1952) by means of diazotation (target cells = sheep erythrocytes) The product is designated 'Sulph SRBC Passive haemagglutination titres of anti Sulph RSA and antiSulph BGG using Sulph SRBC could be inhibited completely by Sulphtyr, Sulph RSA and Sulph BGG in appropiate concentrations

Hapten carrier specific passive haemagglutination was carried out according to *Rubin* (1970), using bisdiazotized benzidine as coupling reagent and SRBC as target cells The products are designated "Sulph-RSA SRBC' and BGG SRBC" Passive haemagglutination titres of antiSulph RSA using Sulph RSA SRBC could be inhibited completely by Sulph RSA but not by Sulphtyr (see also results)

The avidity of antiSulph antibodies was determined by a haemagglutination inhibition test Each antiserum was titrated in absence and in presence of different amounts of Sulphtyr (dose range, 10^{-5}–10^{-9} molar) and the titre against Sulph SRBC was determined The concentration of Sulphtyr which inhibited the Sulph titre 50 per cent was taken as a measurement af avidity

Rabbits were immunized primarily with a single subcutaneous (s c) injection of 5 mg hapten carrier complex emulsified in complete Freund's adjuvant (FCA) Secondary injections of 5 mg antigen were given intravenously (i v) Rabbits were bled from the car vein and blood samples were heat inactivated for 30 min at 56° C

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[1] Abbreviations RSA, rabbit serum albumin, BGG, bovine gamma globulin Sulph, sulphanyl group, Sulphtyr, sulphanyl group coupled to N-chloroacetyl tyrosine

TABLE 1 *LOG_2 Haemagglutination Titre Against Hapten and Carrier Specific Determinants*

Antisera	Sulphtyr*	Days after challenge					
		28		70		97§	
		Sulph SRBC	$Sulph_{11}$RSA SRBC	Sulph SRBC	$Sulph_{11}$RSA SRBC	Sulph SRBC	$Sulph_{11}$RS SRBC
ti$Sulph_{11}$RSA non abs	0	4	8	6,5	11	8	11
ti$Sulph_{11}$RSA non abs	+	1	8	0	10	5	11
ti$Sulph_{11}$RSA absorbed	0	0	8	0	9	0	10
ti$Sulph_{11}$RSA absorbed	+	0	8	0	9	0	10

* Conc of Sulphtyr used, 10^{-4} molar
§ 7 days after rechallenge

TABLE 2 *LOG_2 Haemagglutination Titre Against Hapten and Carrier Specific Determinants*

Antisera	Sulphtyr*	Days after challenge					
		28		70		97§	
		Sulph SRBC	BGG-SRBC	Sulph SRBC	BGG-SRBC	Sulph SRBC	BGG-SRBC
nti$Sulph_{10}$BGG non abs	0	6	8	7	10	8,5	11
nti$Sulph_{10}$BGG non abs	+	0	8	0	10	0	11
nti$Sulph_{10}$BGG absorbed	0	0	8	0	10	0	11

* As Table 1
§ As Table 1

Results and Conclusions

The antibody production to Sulph coupled to BGG exceeds the antibody production to Sulph coupled to RSA during the first 70 days of immunization From this time onwards and 7 days after rechallenge with 5 mg of homologous antigens, the immune responses to the two Sulph complexes did not differ significantly The cooperation hypothesis (*Mitchison* 1970) assumes that an antigen becomes more potent if recognized via carrier determinants Since at the same time the inducing determinant (in this case the Sulph group) must also be recognized at least two receptors are assumed to operate An explanation of the delayed immune response to Sulph when coupled to RSA may be the possible lack of carrier determinants in the Sulph RSA complex

In order to test this hypothesis, the following absorption experiments were carried out anti Sulph RSA sera taken 28 and 70 days after challenge and 7 days after rechallenge (on day 90) were [illegible]
St[illegible]
it [illegible]
could be absorbed completely, 2) that new antigenic determinants were introduced in the RSA molecule during diazotation and 3) that antibodies against the new antigenic determinants were not absorbed with Sulph SRBC The haemagglutination titre against Sulph RSA SRBC of absorbed sera could be inhibited with free Sulph RSA Antibodies against the new antigenic determinants could also be demonstrated by means of the double diffusion method AntiSulph RSA sera absorbed with Sulph SRBC formed precipitation lines towards Sulph RSA and not against Sulph BGG

As the Sulph RSA complex contained new antigenic determinants the immunogenicity of these groups was compared with the immunogenicity of the BGG molecule (*Havas* 1969) Table 1 and 2 shows that the haemagglutination titres against the two carriers were similar during the experimental period which indicate similar immunogenic potency of the two carrier molecule It seems reasonable to assume that the new antigenic determinants are situated near the Sulph group, since these "new" protein structures are introduced either directly through the coupling reaction or indirectly through electrostatic forces (the Sulph group has a highly negative charge) Thus the main structural difference between the RSA and BGG molecule as carriers is that the BGG molecule possesses both proximate and distant carrier determinants, whereas the RSA molecule possesses

only proximate carrier determinants We assume therefore that the immune response to Sulph RSA is delayed because of a lack of distant carrier determinants

Primary immunization with the doses of Sulph-RSA and Sulph-BGG used in the present experiments causes different dependence on carrier A secondary response against Sulph could be elicitated by both Sulph RSA and Sulph BGG in rabbits challenged with Sulph RSA, whereas a secondary response against Sulph in rabbits challenged with Sulph BGG could be elicitated only with Sulph BGG (*Rubin*, unpublished results) Studies of the avidity of anti Sulph antibodies synthesized through stimulation with Sulph RSA and Sulph BGG revealed no difference between the two antibody populations

Finally, earlier experiments in the hapten carrier field (see *Katz et al* 1970) have not distinguished between two types of helper cells reactive against a) native carrier determinants (proximate and distant to the hapten) and b) new antigenic determinants on the carrier (in the proximity of the hapten) It is evident from the present experiments that the latter type of carrier determinants can perform the entire helper function Preliminary results mentioned above indicate that the new proximate determinants lead to a lower degree of dependence on carrier This phenomenon may be due either to similarity of the new antigenic determinants introduced in the different carriers or to the development of a cell compartment almost independent of carrier (antibody forming precursor cells with receptors of high affinity for the hapten)

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Acta path microbiol. scand. Section B. 79, 129–136, 1971

SYNERGISTIC EFFECT IN VIRAL-BACTERIAL INFECTION

3 Histopathological Changes in the Trachea of Mice Following Viral and Bacterial Infection

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The effect of infection with parainfluenza 1 virus or *Haemophilus influenzae* b on the mucosa of respiratory tract of mice was studied. A section of trachea just above the bifurcation was examined histologically for the presence of destructive changes and leukocyte accumulation in the epithelium and lamina propria. Polymorphonuclear (PMN) leukocyte accumulation was a prominent sign after both viral and bacterial inoculation. Viral inoculation produced more frequent and more pronounced inflammatory changes than bacterial inoculation. Disruption of the basement membrane was observed only following virus inoculation. Gross evidence of pneumonia was seen more frequently among virus inoculated animals. The histological changes were progressive with time of survival up to the third day after inoculation. The bacterial titer in homogenates of lung tissue correlated with the number of lobes affected with pneumonia and to some extent with the accumulation of PMN leukocytes in the tracheal epithelium. Bacteria, normally present in the upper respiratory tract, were isolated from a high proportion of virus inoculated lungs. The findings are compatible with the concept that parainfluenza 1 virus infection facilitates a secondary bacterial invasion because of functional impairment or destruction of the epithelium.

Information about the development of virus-induced pathological changes in the respiratory tract has been obtained mainly from experimental infection of laboratory animals (2, 3, 8, 9, 16, 20). However, there is still need for more information about the morphological changes in the respiratory mucosa in relation to different aspects of the infectious process, such as development of pneumonia, presence of infectious agents in the lungs and outcome of the clinical disease. It is also of interest to obtain more information about the interaction between viral and bacterial agents in the development of infection in the respiratory tract.

Our previous studies indicated that intranasal inoculation of mice with parainfluenza 1, Sendai virus and *Haemophilus influenzae* b results in a synergistic effect as defined by enhanced mortality. The primary virus inoculation facilitates a secondary bacterial invasion by reducing the efficiency of defence mechanisms of the host and delaying the elimination of bacteria (5, 6).

The purpose of the present study was to examine the morphological changes in the tracheal mucosa of mice following inoculation with parainfluenza 1 Sendai virus and *H. influenzae b*, and furthermore, to examine whether the morphological [illegible]

Received 4.ix.70

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plain the reduced rate of elimination of bacteria from the lungs after viral inoculation

MATERIAL AND METHODS

Mice HaM/ICR/CSE/Bom albino mice, 3 5 months old, weighing 20–25 g of either sex were used Regularly repeated tests for the presence of haemagglutinating virus and pneumonia virus of mice were all negative

Virus Parainfluenza 1 virus Sendai strain, as previously employed (5 6) was propagated in the allantoic cavity of embryonated eggs 9–11 days old Virus was titrated in embryonated eggs and by the haemagglutination (HA) method using 0 5 per cent guinea pig erythrocytes and veronal buffer as diluent The test was done on plastic trays in a total volume of 1 ml The results were read after 18 hours incubation at 4° C The infectious titer (EID_{50}) was calculated according to the method of *Reed* & *Muench* (18) Aliquots were kept at —18° C Appropriate dilutions for inoculations were made in Hanks basal salt solution (BSS)

Bacteria Haemophilus influenzae b 51 strain was used after 42 passages on solid medium as in previous studies (5) A large number of samples, 1 ml each were frozen in Levinthal broth and kept at —18° C For inoculation a sample was incubated in 5 ml Levinthal broth for 18 hours at 37° C, then centrifuged at 3500 rpm for 10 minutes and reconstituted in physiological saline

Selection and infection of mice All inoculations were done simultaneously by the intranasal route (5 6) and the groups were kept under identical conditions

In the first experiment 90 mice were randomly selected into three groups 30 mice in each One group was inoculated with virus one group with bacteria and one group was given control fluid Parainfluenza virus 10 EID_{50} and *H influenzae* 1–3 × 10^7 colony forming units (CFU) were used as infectious agents and Hanks BSS was used as control in a total volume of 0 1 ml each Four days after inoculation the animals were sacrificed by cervical fracture The groups were reduced during the experiment to 26 animals in two groups and to 25 in one group because of mortality and technical failures

In a second experiment a group of 60 mice were inoculated with virus for study of the development of histological changes with time Ten of these were sacrificed each day up to 5 days after inoculation Ten mice died during the 6 day long period these animals were excluded from the study

Examination of the lungs Lungs were removed and examined for macroscopical signs of pneumonia Deep red infiltrated areas on the surface of varying size could be easily distinguished from the normal lung surface Such areas have been histologically verified as pneumonia in previous studies (6) The extent of changes was quantitated by counting the number of lobes affected

Histological techniques The trachea was removed fixed in 10 per cent formaldehyde for histological examination and embedded in paraffin. Transverse sections of the whole tracheal wall just above the bifurcation were stained with hema [illegible] (PAS)

pendently without knowing which treatment the animals had received The following morphological changes were recorded 1) the presence of leukocytes in the epithelium and lamina propria, 2) disruption of the basement membrane of the epithelium The two investigators disagreed in about 15 per cent of the sections on the extent of leukocyte accumulation, these cases were discussed and a concensus obtained prior to the break of the code

Recovery of the infectious agents The lungs were homogenised with a motor driven teflon tissue grinder in 1 ml Hanks BSS The homogenate was centrifuged at 600 rpm for 10 minutes and the supernatant examined for the presence of *H influenzae* Appropriate dilutions were plated on chocolate agar and bacterial colonies were counted after incubation for 18 hours at 37° C The cultures were also examined for presence of other bacteria than *H influenzae* In many cases large numbers of bacteria could be demonstrated These bacteria were mostly α hemolytic streptococci corynebacteriae and *Staphylococcus albus* the most usual members of the normal resident flora of the upper respiratory tract These contaminating bacteria were not quantitated

The remaining supernatant of homogenate was centrifuged at 3500 rpm for 10 minutes The presence of parainfluenza virus was demonstrated in the supernatant by the HA method

Statistical methods The data were analysed by the χ^2 test and the 2 × 2 table variant of the same test using Yates correction

RESULTS

Normal mouse trachea is lined by a columnar ciliated epithelium (Fig 1) The nuclei are localised in one or several rows in the basal part of cells Normally the lamina propria contains a few leukocytes

The histological changes had similar appearance with patchy distribution in the respiratory mucosa whether the mice were given virus or bacteria (Fig 2) In some areas tall and low cells alternated without

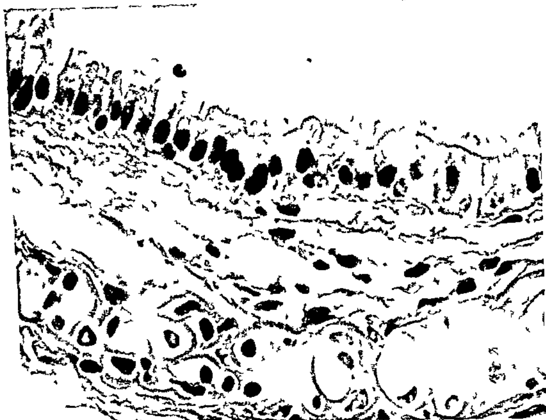


Fig 1 Normal ep thelium from trachea of mice

any regular pattern Tl e cytoplasm in these areas was often granular and contained many vacuoles The nuclei were swollen and scattered through the epithelium without the regularity observed in healthy mucosa In some areas tl e epithelium was desquamated Tl e basement membrane was d srupted in the most severely affected areas of virus inoculated mice (Fig 3) PMN leukocytes had regularly invaded the infected epithelium and often also the lamina propria Large numbers of leukocytes red cells and cellular debris were frequently seen in the lumen of tl e trachea

Quantitative Evaluation of the Infectious Effect on the Respiratory Mucosa

The first experiment showed that accumulation of leukocytes within the epithelium was usually accompanied by accumulation within the lamina propria The number of animals with PMN leukocyte accumulation in the different groups is shown in Table 1 Significantly more of the infected mice showed leukocyte accumulation than the controls ($p < 0\ 001$) The difference between the viral and bacteria inoculated groups was also statistically significant ($p < 0\ 01$)

Only virus inoculated animals showed disruption of the basement membrane (7 out of 26 mice) In these cases we also observed particularly severe PMN leukocyte accumulation in the lamina propria

The virus inoculated animals showed macroscopical pneumonia in a higher proportion than bacteria inoculated animals ($p < 0\ 05$) (Table 2) The presence of inflammatory changes in the trachea correlated positively with the presence of gross evidence of pneumonia in all three groups

9*



Fig 2 Tracheal section from mice inoculated with parainfluenza 1 virus showing disorganized epithelial lining and cell infiltration (× 400)



Fig 3 Tracheal section from mice inoculated with parainfluenza 1 virus Disruption of the basement membrane (× 400)

TABLE 1 *Leukocyte Accumulation in the Tracheal Epithelium and the Lamina Propria in Mice Inoculated with Parainfluenza 1 Virus* H influenzae *or Culture Medium*

Material inoculated	Number of mice	Number and per cent* of mice with PMN leukocytes in: Epithelium	Lamina propria
Virus	26	21 (79)	23 (87)
Bacteria	25	13 (52)	16 (64)
Culture medium	26	4 (155)	5 (21)

* Per cent in parenthes

Correlation between Pathological Changes and the Titer of Infectious Agents in the Lungs

There was no correlation between virus titer in the lungs and the morphological changes On the other hand the titer of *H influenzae* in the lungs of bacteria inoculated mice correlated positively with the presence of inflammatory changes in the tracheal mucosa (Table 3) and with the number of lobes affected with pneumonia (Fig 4) Contaminating bacteria were most frequently isolated from the lungs of virus inoculated animals (12 out of 14), less frequently from the bacteria inoculated (4 out of 15) and from none of the control mice

TABLE 2 *Gross Evidence of Pneumonia in the Lungs of Mice Inoculated with Parainfluenza 1 Virus, H influenzae or Given Culture Medium*

Material inoculated	Pneumonia* present	Pneumonia* absent	
Virus	19	2	p<0 05
Bacteria	13	8	
Culture medium	2	22	

* Macroscopical appearance of the lungs was not recorded from 11 mice

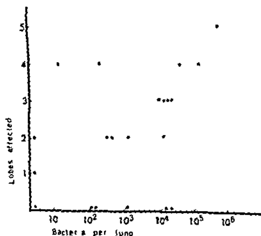


Fig 4 Correlation between the lobar extent of pneumonia and the titer of *H influenzae* in the lungs of mice, 4 days after bacterial inoculation

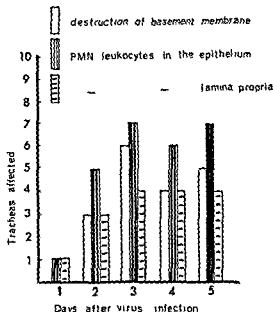


Fig 5 Leukocyte accumulation within the epithelium and lamina propria and destruction of the basement membrane at different survival times after inoculation with parainfluenza 1 virus

Effect of Time after Virus Inoculation on the Morphological Changes in the Trachea

The number of virus inoculated animals with signs of tracheal inflammation and disruption of the basement membrane at different survival times is shown in Fig 5 Leukocyte accumulation and destruction of the basement membrane was found with increas-

TABLE 3 *Bacterial Titer in Homogenised Lung Tissue Correlated with the Presence of Inflammatory Changes in the Trachea Mucosa in Mice Inoculated with H influenzae*

Leukocytes within		Number of mice	Number of mice with H influenzae <10⁴ per lung	Number of mice with H influenzae >10⁴ per lung	
Epithelium	present	13	4	9	*
	absent	12	9	3	
Lamina propria	present	16	8	8	§
	absent	9	5	4	

* $p<0.05$
§ $p>0.1$

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Acta path microbiol scand Section B 79, 137–141, 1971

SYNERGISTIC EFFECT IN VIRAL-BACTERIAL INFECTION

5 *Functional Studies on the Role of the Ciliary Activity in the Mouse Trachea*

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The role of the tracheal ciliary activity in the host defence against bacterial respiratory infections was studied *In vivo* infection of the mouse and *in vitro* infection of the mouse trachea organ cultures with parainfluenza 1, Sendai virus resulted in a patchy inhibition of ciliary activity Lidocain chloride also inhibited reversibely the ciliary activity of organ culture systems in concentrations from 0 3 to 2 mg per ml medium The same concentration had no effect on the phagocytic ability of alveolar macrophages Intranasally inoculated *Hemophilus influenzae* were eliminated at a slower rate from the lungs of Lidocain treated mice than from normal animals during the initial phase of infection Slightly more mice died following *H influenzae* infection after Lidocain treatment than without it but the difference was not significant The data support the concept that Sendai virus infection results in a reduced ciliary activity in the trachea, which again results in a delayed elimination of bacteria from the lungs

Respiratory virus infections apparently influence several defence factors in the infected organism In a previous communication (8) we have described the histopathological changes of tracheal mucosa following parainfluenza 1 virus infection in the mouse The findings were compatible with the concept that the virus infection produces lesions which represent functional impairment of the ciliary epithelium However, no definite conclusions could be drawn regarding the functional role of these lesions In the present study we attempt to further evaluate the role of the ciliary activity Reversible inhibition of clam ciliary activity by the local anesthetic, Lidocain chloride was described by *Krahl & Bulmash* (13) In this study we have employed this agent in order to obtain a selective inhibition of the ciliary activity of the respiratory tract

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MATERIAL AND METHODS

Mice HaM/ICR/CSE/Bom albino mice 3–5 months old, weighing 20–25 g of either sex were used as in previous studies (6, 8)

Virus Parainfluenza 1 virus Sendai strain was produced and titrated as described (7)

Bacteria Haemophilus influenzae b 51 strain was employed Culturing and preparation of the inoculate was done as in previous studies (7 8)

In vivo experiments Lidocain chloride (Xylocain®) 4 per cent solution was purchased from Astra, Sodertalje Mice were given light ether anesthesia then 0 015 ml Lidocain was placed on the nasal openings The animals were held in an

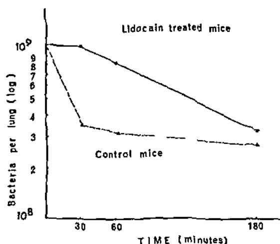


Fig 1 The effect of intranasal Lidocain treatment on the elimination of *H influenzae* from the lungs

Effect of Lidocain on the Phagocytic Ability of Alveolar Macrophages

Alveolar macrophages were obtained from normal mice The pool of cells was distributed equally into several tubes, about 3×10^5 macrophages in each Lidocain was added in different concentrations Parallel samples containing the same medium but no cells were also included in the experiments *H influenzae* was inoculated into all tubes After 1 hour incubation in a tissue culture roller at 37° C samples were removed and the number of surviving bacteria estimated (Table 3) Lidocain had but a slight effect on the viable count within the limit of 1 3 mg per ml concentration The same concentration had *no* significant effect on the phagocytic ability of alveolar macrophages Higher concentrations of the Lidocain were bactericidal for the *H influenzae*

DISCUSSION

An inhibitory effect of different viruses on the ciliary activity of different hosts has been demonstrated by several authors (3 12, 15) In many instances virus multiplication and histopathological lesions developed parallelly with the inhibition of ciliary activity (11) The action of parainfluenza 1, Sendai virus on the mouse ciliary epithelium, the model employed in the present study, has been previously examined by *Willems* (16, 17) Virus multiplication was followed by interferon production, histological changes, and reduction of the ciliary beat Our observations on the effect of Sendai virus on the ciliary activity are comparable with the findings of Willems They are also compatible with our previous data on histological lesions following Sendai virus infection in the respiratory mucosa (8)

With the application of Lidocain we have seemingly succeeded in reproducing the inhibitory effect of virus on the ciliary activity without affecting the other main defence factor, the phagocytic activity of alveolar macrophages However, it should be remembered, that numerous other host defence factors which are not considered in this study, might be influenced by this treatment Therefore conclusions drawn from these data must be graded for these uncontrolled changes which possible accompany the demonstrated alterations

The inhibited ciliary activity results in a delay of elimination of bacteria from the lungs This inhibition seems to be reversible

TABLE 3 *The Effect of Lidocain on the Phagocytic Ability of Alveolar Macrophages from the Mouse*

Lidocain concentration	Colony count after 60 minutes* incubation	
	Macrophages present	Control medium
0	$4\,2 \times 10^5$	$9\,4 \times 10^5$
0 5 mg per ml	$5\,0 \times 10^5$	$7\,9 \times 10^5$
1 3 mg per ml	$2\,6 \times 10^5$	$5\,8 \times 10^5$
4 0 mg per ml	ND	$3\,5 \times 10^3$

* Inoculate $5\,5 \times 10^5$
ND not done

indicated by in vitro experiments, and by the short duration of the delay in the elimination of bacteria The in vivo inhibition of ciliary activity by Lidocain is probably of short duration, however, the exact length cannot be determined from the present experiments. A short duration may explain, that Lidocain treatment was without significant effect on the mortality We know that Sendai virus infection results in an extended inhibition of ciliary activity which probably causes an extended delay in the elimination of subsequent bacterial infection, and possibly a higher mortality Another possible explanation for the uncertain effect on mortality might be, that a selective inhibition of one defence factor is compensated by other mechanisms, and therefore not sufficient to establish a more severe disease with increased mortality

Numerous agents and physiological alterations have been shown to inhibit ciliary activity, such as alcohol, cigarette smoke, formic and acetic acids, pH change and hypoxia (1, 2, 4, 5, 14) Unfortunately this effect is usually not selective, as many of these agents also affect other host defence factors (9) Construction of models with controlled selective inhibition of the different factors for extended periods remains to be solved Only such models can give a definite assessment of the relative role of the different host factors

The present data are in agreement with the generally accepted role of the ciliary activity in the process of elimination of foreign particles from the lungs They also indirectly support the concept that Sendai virus infection inhibits this action, which in turn results in a delayed elimination of bacteria following a secondary infection

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IMMUNOELECTROPHORETIC PATTERNS OF EXTRACTS FROM ALL *ESCHERICHIA COLI* O AND K ANTIGEN TEST STRAINS CORRELATION WITH PATHOGENICITY

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Simple water extracts of all Escherichia coli O antigen *test strains 01–0150* and K antigen test strains K1–K91 were examined in immunoelectrophoresis test The precipitation arcs corresponding to the O antigen specificity and to the thermostable polysaccharide K antigen were easy to identify All strains gave an O antigen precipitation arc found either on the anodic or the kathodic side of the application basin and close to this Only a limited number of strains contained a special thermostable K polysaccharide always negatively charged According to the movement of the O antigen and to presence or non presence of a polysaccharide K antigen the extracts and thus the strains could be divided into a few groups which fitted well with our present knowledge about pathogenicity in different *E coli* strains Serotypes found frequently in normal faeces and in extra intestinal disease had kathodic O antigens and a special negatively charged K antigen The so called enteropathogenic types (from infantile diarrhoea) had a kathodic O antigen and no special K antigen Types from dysentery like disease had a negatively charged O antigen but no special thermostable K antigen Thus *E coli* strains which may invade the tissues when conditions allow have a negatively charged surface antigen either O antigen lipo *polysaccharide or K antigen polysaccharide or both Acidic components most often* hexuronic acids or neuraminic acid were found in side chains from most of the strains with an anodic O antigen

For many years serology has been among the methods of choice for subdivision of bacterial species Historically bacterial agglutination has often been the first method used but in several cases it has been abandoned again because of the development of more precise techniques However in *Enterobacteriaceae* bacteriology the agglutination technique has retained its dominant role The O antigen which is common to all *Enterobacteriaceae* is a complex lipopolysaccharide located in intimate relation to the bacterial cell wall The polysaccharide part of this molecule is

Received 28 viii 70

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responsible for the O specificity It is stable against heat treatment Thus innumerable O antigen determinants can be defined precisely by the agglutination technique, and O antigen determination is the backbone of *Enterobacteriaceae* serology The flagellar antigens, H antigens, can be used with almost the same simplicity and precision for the definition of motile strains, *e g* many *Salmonella* and *Escherichia* strains

In addition to these two antigenic groups, there exists a large heterogeneous family of surface antigens which also contain the so-called K antigens Only few K antigens are described among *Salmonella*, particularly the Vi antigen and M antigen, both of which are polysaccharide in nature In the *Klebsiella* group the polysaccharide K antigens are so well developed that they have been used for years for subdivision into serotypes in this group The agglutination reaction and the capsular quelling reaction are both simple and effective for use with these bacteria because of the strong development of the capsules

In the *Escherichia* group, *Kauffmann* (14) described almost 30 years ago surface antigens that were neither O nor H antigens Together with his co workers, he coined the designation K antigen (K for Kapsel = capsule) and it was found furthermore that different K antigens, A, B and L, could be differentiated (15 16) It was understood that each coli strain contained only one K antigen, even though this fact was not clearly expressed This terminology will not be discussed here, it will only be stated that it has been accepted generally and has been used in the serological subdivision of the *Escherichia* group into serotypes In contrast to this general acceptance of the principles, the practical K antigen determination was never very popular Even though 91 *E coli* K antigens were described, typing of these was never used generally In most cases one relied merely on type determination of O and H antigens In the *Shigella* group, K antigens have also been known to exist for more than 20 years (21), but no one has succeeded in including these antigens in the serological analysis

The authors of the present paper feel that the probable explanation for this state of affairs is simply that the agglutination technique is not sufficient for general determination of K antigens This is partly because the single bacterial organism has many different antigenic substances on its surface, antigens that are not characterized well enough by the agglutination technique

A number of new serological techniques have been described in recent years and some of these have also been applied to *Escherichia coli* antigens The indirect haemagglutination (HA) technique as described by *Neter et al* (22) has been used by *Kunin & Beard* (18) and other authors for the characterization of antigens, though often under conditions which made it difficult to know which type of antigen was involved Generally this HA technique has been used when the search was for antibody response during *E coli* infections The HA method has also been used extensively in immunochemical research on *Enterobacteriaceae* polysaccharide O antigens (see the review by *Luderitz, Staub & Westphal* (19) and on *E coli* acidic polysaccharide K antigens (12, 20)

The double immunoprecipitation in gel method (29) has been used on many occasions for characterization of *Enterobacteriaceae* antigens (1, 9, 11, 27, 33) Immunoelectrophoresis (IE) has been used less extensively (7), but in recent years a number of examinations, especially on *Shigella* antigens (4, 5) have been published *Holmgren, Eggertsen, Hansen & Lincoln* (11), *Grados & Ewing* (8) and *Ørskov & Ørskov* (28) have used this method for characterization of *E coli* antigens

In the last mentioned paper, the IE technique was used for examination of *E coli* K(L) antigens It was shown that the agglutinability of the strains containing L antigen was influenced by heating, while at the same time a heat stable K antigen was eluted from the bacterium Based on these experiences an examination has been carried out in

volving IE of simple extracts from all *E coli* O and K antigen test strains

The result of these investigations is that the many IE results can be ranged into a small number of well defined IE patterns

METHODS

Medium A medium described by *Schlecht* & *Westphal* (31) under the designation D_5 was used in slightly modified form The amount of glucose which in the original medium was 0 3 per cent, was reduced to 0 05 per cent

Strains All *E coli* O antigen test strains O1 to O150 and all *E coli* K antigen test strains K1 to K91 with the exception of K88 were examined As some of the O antigen test strains have been removed and not substituted the total numbers of antigen test strains examined were 146 O and 90 K strains These were all established test strains of the WHO International Escherichia Centre at Statens Seruminstitut Copenhagen

Extraction The bacteria were cultivated on the surface of the modified D_5 medium at 37°C After incubation for 20 hours the growth was suspended into saline Culture from four 14 cm diameter plates was used per 10 ml saline *i e* about 0 3 g wet weight per ml This suspension was heated to 60°C for 20 minutes in waterbath followed by centrifugation at 8000 *g* for 15 minutes These extracts labelled *60° 20*, could be kept for weeks at 4°C Part of this extract was then heated to 100°C for 1 hour and labelled 60° to 100° In some cases equal parts of N N dimethylformamide were added to the *60° 20* extract before heating to 100°C, in these cases the polysaccharides were precipitated after heating by addition of 5 volumes of acetone Centrifugation was performed after precipitation at room temperature overnight The pellet was washed once with acetone and dried at 37°C The acetone dried powder was resuspended in saline corresponding to the original volume As the formamide treatment had no influence on the polysaccharide immunoprecipitations described in this paper it has not been indicated when formamide treated *60° to 100°* extracts were used

Sera Rabbit O and OK sera most of them routine sera from the WHO International Escherichia Centre were used These were produced according to traditional procedures (6 17)

The immunoelectrophoretic technique was that of *Scheidegger* (30), using the apparatus manufactured commercially by LKB of Sweden Three glass slides were coated with 9 ml 1 5 per cent agar Noble in veronal buffer pH 8 6 ionic strength 0 05 *μ*. Wells and troughs were cut with the LKB device The agar from the troughs was sucked off and the troughs filled with 1–1 5 μl antigen solution Veronal buffer pH 8 6 ionic strength 0 05 *μ* was used in the electrophoretic chamber The electrophoretic separation was performed at a voltage of 7 V/cm for 2 hours The agar in the well was lifted out and the well filled with about 150 μl undiluted antiserum The slides were left in a moist chamber overnight Before staining the results were recorded graphically by drawing The slides were washed for 2 days in saline and on the 3rd day washed in distilled water before staining with Amidoschwarz

Isolation and degradation of lipopolysaccharides as well as *analytical methods* have been described previously (13, 12)

RESULTS

The main conclusion that can be drawn from the examination in immunoelectrophoresis (IE) of all *E coli* O and K antigen test strains is that the many individual patterns found can be placed into a limited number of different groups Virtually, only two different patterns are found with O serum (Fig 1), *viz* pattern 1 (subdivided into 1A and 1B) where the O antigen has moved towards the cathode, and pattern 2 where the O antigens have moved towards the anode *E coli* O antisera contain no other precipitins easily detectable in IE than those directed against the O antigen As the test is carried out here the sensitivity is such that the extracts used will rarely show other lines than the one mentioned The fact that this line is actually caused by precipitation of the O antigen can be confirmed by the reaction of identity when extracts from different strains with the same O antigen but with different K antigen are compared Such strains are at present primarily found among strains having O groups which belong to IE group 1 (Fig 1) Absorption of an O or an OK serum with boiled culture of the homologous strain or of a heterologous one with the same O antigen will remove this line The O precipitation line is practically always situated close to the application basin either on one side or the other In some cases two or three parallel precipitation lines are found where the O antigen is situated A similar duplication of the O antigen precipitation line is well known from investigations

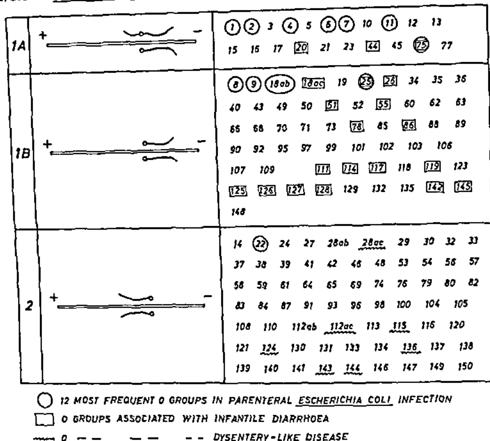


Fig 1 Immunoelectropherogram In the trough homologous O antiserum In basin above trough 60° 20' extract In basin below trough 60° to 100°C extract (see text) Numbers in right hand column are O antigen numbers

using the Ouchterlony technique The explanation for this phenomenon is not known, but might be due to the possible existence of different classes of size among the lipopolysaccharide molecules

The fact that some O antigens or lipopolysaccharide molecules move towards the anode and others towards the cathode is unquestionably caused by differences in electrical potential of the molecules, but the factors which determine this difference are not known Recently about one hundred *E coli* O antigen test strains—a hundred of the same strains included in this study—were examined for the sugar composition of the polysaccharide part of the lipopolysaccharide (25) A comparison of the results of that study and the present examination shows that the majority of the O polysaccharides with few basic sugars (chemotypes I and II) belong to the ones moving towards the anode (IE pattern 2) Chemical analysis of these lipopolysaccharides showed that most of them contain either hexuronic acid or N acetyl neuraminic acid In one case glycerol phosphate was found (13) This is shown in Table 1 It was of course important to demonstrate that this is not due to contamination of the lipopolysaccharides with an acidic polysaccharide, also produced by the strain in question Therefore the lipopolysaccharides were repeatedly centrifuged at high speed—in some

TABLE 1 *Acidic Components Found in Lipopolysaccharides of E. coli*

Acidic component	O antigen
Hexuronic acid	22 27 32 33 37 41 46 48 53 54 57 58 59 64 65 74 76 79 80 83 87 91 96 105 110 112ac 113 115 116 120 124 133 134 139 140 141 143
N acetyl neuraminic acid	24 56 61 136
Glycerol phosphate	100

cases as many as 8 times Then they were degraded with 0 1 N acetic acid and the degraded polysaccharides were fractionated on Sephadex (for details see 32) Using this method the O specific polysaccharide moiety of the lipopolysaccharides can be studied separately In all cases analysed the values of the acidic components were much higher in the O specific polysaccharide fraction than in the complete lipopolysaccharide Representative values found in *E coli* 0141 K85 are 18 per cent and 2 per cent respectively This is due to the fact that frequently lipopolysaccharide complexes contain only few O specific polysaccharide chains One such antigen 0100 belonging in IE group 2 contains phosphate groups responsible for this capacity (13)

In groups 1A and 1B the arc stretching from the origin towards the cathode may result from endosmosis Therefore this migration may not necessarily be due to basic groups in the O antigen Endosmosis would neither affect the principle of classification into IE groups 1 and 2 nor the subdivision of group 1 into 1A and 1B If the cathodic migration of the O antigen in group 1 is only due to endosmosis then anodic mobility of the little O arc in group 1 and the charged O antigen in group 2 is greater than apparent on the immunoelectropherograms

Group 1 has been subdivided into two subgroups 1 A and 1 B based on a small precipitation line on the anode side of the application basin Characteristically this extension of the O line is only found in the *60° 20* extract but against both O and OK sera This is an O specific line the heat lability of which is difficult to explain Possibly association of the O antigen to a negatively charged moiety is broken irreversibly by heating to 100°C This may be the effect of heat denaturation and the charged moiety may then be a protein The phenomenon is investigated further

All the O antigen test strains established hitherto are listed in Fig 1 An attempt has been made to correlate some other characteristics of these strains with the IE groups presented here

Frequency of O Antigens

It is a well known fact that some coli O groups are far more common than others and that the same frequent O groups are found more often in pathological material The 12 most frequent O groups were selected from a recent investigation of 560 *E coli* strains from human septicaemia cases (26) It will be seen from Fig 1 that practically all these groups belong in category 1 and especially 1A One exception is O group 22 It can be mentioned that the 022 strain has a thermostable negatively charged K antigen (see Fig 2)

Infantile Diarrhoea

In Fig 1 the O groups of (enteropathogenic) serotypes claimed to be involved in outbreaks of infantile diarrhoea have been marked It is apparent that they all fall into

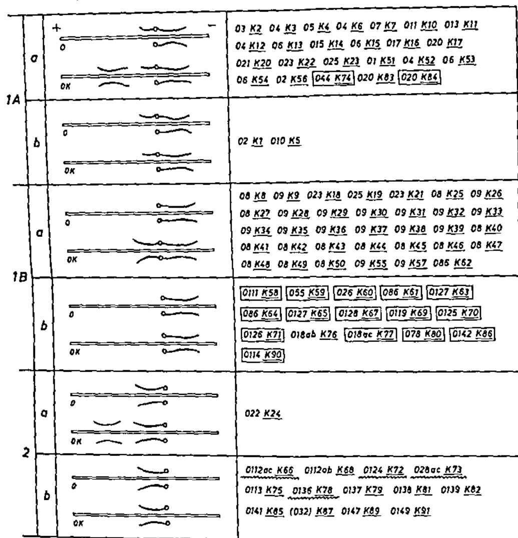


Fig 2 Immunoelectropherogram In the trough homologous O or OK antiserum In the basin above trough 60° 20' extract In the basin below trough 60° to 100°C extract (see text)

group 1 and most of them into group 1B a fact which, according to the above mentioned hypothesis, would indicate that they had no special K antigen polysaccharide This suggestion is confirmed by the examination of these strains in OK sera, which are shown in Fig 2 There they can all be grouped in IE group 1Bb

„Dysentery-like" Disease

In recent years, another but different group of *E coli* strains has been interpreted as being associated with dysentery-like disease in adults and children This group of *E coli* serotypes should be kept separate from the so-called enteropathogenic *E coli* of infantile diarrhoea, as has already been

TABLE 1 *Acidic Components Found in Lipopolysaccharides of* E coli

Acidic component	O antigen									
Hexuronic acid	22	27	32	33	37	41	46	48	53	54
	57	58	59	64	65	74	76	79	80	83
	87	91	96	105	110	112ac	113	115		
	116	120	124	133	134	139	140	141		
	143									
N acetyl neuraminic acid	24	56	61	136						
Glycerol phosphate	100									

cases as many as 8 times Then they were degraded with 0 1 N acetic acid and the degraded polysaccharides were fractionated on Sephadex (for details see 32) Using this method the O specific polysaccharide moiety of the lipopolysaccharides can be studied separately In all cases analysed the values of the acidic components were much higher in the O specific polysaccharide fraction than in the complete lipopolysaccharide Representative values found in *E coli* 0141 K85 are 18 per cent and 2 per cent respectively *This is due to the fact that frequently lipo* polysaccharide complexes contain only few O specific polysaccharide chains One such antigen 0100 belonging in IE group 2 contains *phosphate groups responsible for this* capacity (13)

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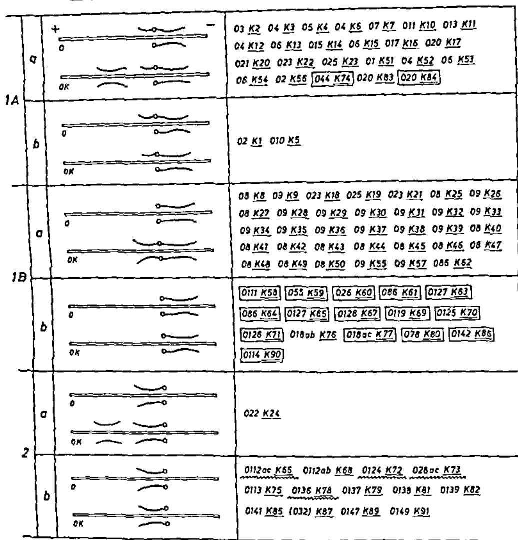
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OK GROUPS ASSOCIATED WITH INFANTILE DIARRHOEA

DYSENTERY-LIKE DISEASE

Fig 2 Immunoelectropherogram In the trough homologous O or OK antiserum In the basin above trough 60° 20' extract. In the basin below trough 60° to 100°C extract (see text)

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Acta path microbiol scand Section B 79 153–162, 1971

IN VITRO STUDIES ON THE FATE OF ANTIGEN

4 *The Digestion of Human Serum Albumin and Ferritin by Extracts of Mouse Spleen*

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Extracts of mouse spleen and mouse peritoneal macrophages digested human serum albumin (HSA) at pH 3 0 *in vitro* the products of digestion gave three different precipitating components as shown by immunoelectrophoresis The extent to which ^{125}I HSA was degraded could also be followed by assaying the radioactivity on a paper strip after zone electrophoresis of the HSA digests The presence of ferritin did not affect the digestion of HSA Ferritin was not cleaved by the spleen or cell extract at pH 3 0, 4 5 or 8 0, even after incubation at 37°C for 24 hours These results are discussed in relation to the fate of the antigens *in vivo*

Lapresle et al (1959) have conclusively established that human serum albumin (HSA) is digested by rabbit spleen extract, *in vitro* During the first stages of proteolysis, the complex antigenic structure is split into three distinct products which precipitate with three different antibodies present in the serum of rabbits immunized with undigested albumin It was of interest to ascertain whether extracts of mouse spleen and mouse peritoneal macrophages would degrade HSA in a similar manner to rabbit spleen extract

It has been shown previously *(Rhodes & Lind 1968)* that HSA and ferritin are engulfed concurrently by the same peritoneal cells from normal mice and those undergoing primary and secondary responses There was therefore, no indication that one antigen was ingested in preference to the other in any of the groups of mice However, it is possible that a selection could take place intracellularly Evidence has been presented (*Uhr & Weissman* 1965) that a necessary step in the synthesis of antibody is the degradation of antigen by lysosomes in the granules of the reticulo endothelial cells If HSA and ferritin were degraded at different rates within lysosomes then one antigen or antigenic determinant might induce the formation of specific antibody before the other antigen It would be interesting to know whether this is possible, in view of the fact that ferritin, in saline, is a stronger immunogen in mice than HSA (own observations) Experiments were carried out to determine the extent of digestion of HSA and ferritin after incubation with extracts of mouse spleen and mouse peritoneal macrophages The products of the digestion of radioactively labelled HSA and

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hours' incubation with spleen extract at 37°C (see Fig 5)

All the experiments described so far were repeated, using mouse macrophage extract yielding results similar to those mentioned above Since ferritin appeared to be stable under these conditions, it was of interest to test the susceptibility of the protein component of ferritin, *i e* apoferritin to digestion by spleen extracts

Digestion of HSA, Ferritin and Apoferritin at Different pH Values

The proteins were incubated at 37°C with spleen extract at pH 3 0, 4 5, and 8 0 for various periods of time The incubation mixtures were adjusted to pH 7 0 for HSA, pH 8 0 for ferritin and pH 8 0–9 0 for apoferritin after incubation, centrifuged and the supernatants were examined by IE The results of these experiments indicated that *HSA* was only digested at pH 3 0 *Ferritin* was not digested at any of the pH values up to 24 hours' incubation All the controls (*i e* no spleen extract present) for *apoferritin* showed two to three lines of precipitation at 0 hours irrespective of the pH to which the protein was exposed A part of the apoferritin appeared to be digested at pH 3 0 since one line of precipitation had disappeared at 24 hours after incubation

DISCUSSION

Extracts of mouse spleen and mouse peritoneal macrophages were shown to digest HSA in the same manner as rabbit spleen extract, *i e* in the early stages of proteolysis HSA is split into three polypeptides which precipitate with anti HSA rabbit serum The digestion occured only at pH 3 0 and not at 4 5 or 8 0, which is in agreement with the findings for rabbit spleen extracts (*Lapresle et al* 1959)

Under these experimental conditions ferritin is resistant to proteolysis even up to 24 hours' incubation with spleen extract or macrophage extract In contrast the protein component of ferritin apoferritin, consistently showed the presence of 2–3 polypeptides when tested in IE, even in the absence of enzyme at all pH values tested *i e* 3 0, 4 5 and 8 0 *Suran & Tarver* (1965) demonstrated that both ferritin and apoferritin contained 3–5 analogous fractions upon electrophoresis in starch or acrylamide gels at pH 8 6 Electrophoresis at pH 3 0 showed the same number of components to be present. The finding that apoferritin contains three fractions in IE after digestion at pH 3 0 and 8 0 is consistent with the above observations

A feasible explanation of the fact that ferritin is not digested in the presence of spleen extract is that the iron micelle might stabilize not only the apoferritin to which it is reversibly attached but also the 20–25 per cent free apoferritin which is present in ferritin (*Granick* 1946) In fact it was found by *Crichton* (1969) that the presence of the iron micelle in ferritin considerably reduced the extent to which the protein was digested by trypsin

These *in vitro* findings, whilst indicating that HSA and ferritin are digested at different rates by spleen and macrophage extracts may have no significance *in vivo* However it is known that some of the different types of cells with which HSA and ferritin would come into contact *in vivo*, such as macrophages and polymorphonucleocytes in the circulation and in the tissues do contain many hydrolases including cathepsin (*Cohn & Benson* 1965) Cathepsin was found by *Lapresle et al* (1959) to be the enzyme in spleen extract which was responsible for the degradation of HSA at pH 3 0 Despite the fact that enzymes are available for the digestion of HSA *in vivo* it has not yet been demonstrated that the protein is actually degraded into three different polypeptides *in vivo* However *Lapresle & Goldstein* (1969) have isolated an inhibitor from the digestion products of HSA *in vitro* This inhibitor when degraded with trypsin gave rise to a smaller immunologically active fragment designated F1 Immunization of rabbits with F1 induced the formation of antibodies which reacted with the native protein F1 contained only

one of the antigenic determinants of the albumin molecule, and had a molecular weight of 6600

Nevertheless, the rapid digestion of HSA *in vitro* might correlate with what is known about the fate of HSA *in vivo*

1 HSA is eliminated very rapidly after intraperitoneal injection in mice, and is degraded rapidly by peritoneal macrophages (*Rhodes* 1970)

2 HSA is trapped and retained very inefficiently in rat lymph nodes in contrast to stronger immunogens such as flagellin, and in contrast to HSA|anti HSA complexes (*Ada & Lang 1966*) HSA is also very inefficiently trapped in mouse lymph nodes and in mouse spleen (*Rhodes*, unpubl observations) However, it is not yet proved that trapping of antigen is synonymous with antibody formation

Soluble ferritin is a somewhat stronger immunogen than HSA in mice (own observations) It is known that ferritin is taken up by the phagocytic cells in rabbit lymph nodes (*Wellensiek & Coons* 1964) and in the medullary macrophages and the dendritic macrophages of the follicules in mouse spleen (own observations) Little is known about its actual fate in these organs, although *Buynkover et al* (1965) appear to have evidence of digestion of ferritin in vesicles in the reticular cells of the rat lymph node

Recently, a pertinent observation on the degradation of antigens by mouse liver lysosomes was made by *Ryan & Lee (1970)* They degraded various antigens *in vitro* and the extent of this degradation was correlated with the immunogenicity of the antigens *in vivo* The authors concluded that proteins which were unable to elicit an antibody response in the mouse were extensively hydrolysed by lysosomal proteins These results support those found in the present experiments HSA is a poor immunogen in the mouse and it is degraded rapidly, whereas ferritin which is a stronger immunogen than HSA in the mouse is not degraded by extracts of mouse spleen or mouse macrophages

Further experimentation on the rat of degradation of HSA and ferritin *in vivo* and on the products of such a degration are required before the *in vitro* findings can be correlated with what actually occurs *in vivo*

I am greatly indebted to cand pharm *B Mansa* for critical advice and help in compiling the manuscript

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TABLE 1 *Australia SH Antigen and Antibody in Serum of Patients with Liver Disease*

Patients with	Number of patients				
	Australia SH antigen		Australia SH antibody		Total
	present	absent	present	absent	
Infectious hepatitis	0	35	0	35	35
Serum hepatitis	2	1	0	3	3
Possible serum hepatitis	7	3	0	10	10
Chronic hepatitis	1	2	0	3	3
Occlusive jaundice	0	19	0	19	19
Infectious mononucleosis	0	6	0	6	6
Toxic hepatitis	0	8	0	8	8
Total	10	74	0	84	84

Table 1 Antibody to the Australia-SH antigen was not demonstrated in any of the serum samples obtained from the patients

The group scored as infectious hepatitis comprises 35 patients, none of whom were known to have been inoculated during the last six months before the onset of the disease No patient in this group had Australia-SH antigen in the serum

Two of the three patients scored as suffering from serum hepatitis were drug addicts, taking injections of narcotics, while the third had received multiple blood transfusions three months prior to the onset of hepatitis The two addicts had Australia SH antigen in the serum, whereas we could not demonstrate it in serum of the last patient

The group "possible serum hepatitis' comprises ten patients, all of whom had been exposed to vaccinations, injections, blood sampling or dental treatment one to six months before the manifestation of the disease Sera from seven of the ten patients contained the Australia-SH antigen

Three patients suffered from chronic hepatitis (diagnosis confirmed by liver biopsy) The Australia SH antigen was demonstrated in serum of one of them

The Australia SH antigen was not found in the serum of any patient belonging to any of the last three groups in Table 1 The six patients with infectious mononucleosis had *simultaneous* jaundice The group "toxic hepatitis" includes eight patients suffering from severe infections, intoxications with jaundice or chronic alcoholism with acute exacerbations of the liver disease

This work was supported in part by grants from the *Norwegian Research Council for Science and the Humanities*, and from *Nordisk Insulinfond*

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HUMAN SECRETORY IMMUNOGLOBULINS

3 Immunochemical and Physicochemical Studies of Secretory IgA and Free Secretory Piece

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Human secretory IgA is mainly a disulfide bonded dimer whose polymer conformation is secondarily stabilized by conjugation with one molecule of 'secretory piece' (SP) The noncovalent interactions between SP and IgA are highly dependent upon an apparently variable number of disulfide bridges between the same units, and are responsible for a packing of the composite molecule to a degree that the most immunogenic region of SP is masked This inaccessible (I) determinant, as well as one of its accessible (A_1) antigenic groups is extremely susceptible to reduction alkylation Antigenically intact SP can therefore hardly be obtained by degradation of secretory IgA The released molecules are of the same size as native free SP, but a proportion of them may have only one (A_2) or two (A_2 and A_1) unimpaired determinants Maximum release of SP with three reactive determinants occurs after reduction with 5 mM dithioerythritol, 0 075 M β mercaptoethanol, or 0 05-0 075 M cysteine HCl—the first two followed by alkylation

Secretory immunoglobulin A (IgA) is unique in that, in addition to light and heavy polypeptide chains, it contains an epithelial glycoprotein—the so called 'secretory piece" (SP) Several structural models have been proposed for the composite molecule Two units of monomer (7S) IgA and two of SP apparently constitute the rabbit variety (*Cebra* 1969), three units of 7S IgA and one of SP (*Hong et al* 1966)— or one 7S IgA and several SP units (*Tomasi & Bienenstock* 1968)—have been proposed for the human counterpart The majority of available evidence (*Newcomb et al* 1968, *Tomasi & Czerwinski* 1968, *Hurlimann et al* 1969) indicates, however, that the human 11S component is composed of one SP and two 7S IgA molecules, although data about the forces stabilizing its quaternary structure have been conflicting Moreover, there are two minor populations of larger secretory polymers whose composition is completely unknown (*Brandtzaeg et al* 1970)

A recent model (*Brandtzaeg et al* 1970) included several previously undefined antigenic characteristics, and indicated the interunit bonding of human secretory 11S IgA It visualized a preformed IgA dimer secondarily stabilized by covalent and noncovalent interactions through conjugation with SP The model was to a great extent based on experiments described in the present publication and preliminary findings reported elsewhere (*Brandtzaeg* 1968, 1970)

MATERIALS AND METHODS

Human Serum and Secretions

The samples of normal human serum (NHS), colostrum and parotid secretions (ps) were those used in a preceding study (*Brandtzaeg et al* 1970)

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TABLE 1 *Australia-SH Antigen and Antibody in Serum of Patients with Liver Disease*

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Wright, R, McCollum, R W & Klatskin G Australia antigen in acute and chronic liver disease Lancet *II* 117–121, 1969

Fig 11A

Fig 11 (A B and C) DEAE ... of colostral IgA before and after reduction ... reduced alkylated colostral IgA Column size ... Samples were in A 8 8 mg of colostral IgA ... in B 8 8 mg of colostral IgA reduced with ... and alkylated, and in C concentrated pool of fractions 60 70 (5 6 ml OD 280 nm = 0 33) from experiment ... M phosphate buffer pH 7 5 contain... ensity (OD) of fractions Below Pr... after step (except 1 0 M NaCl) as revealed by SRID with antisera to light (L) or heavy (μ γ and α) immunoglobulin chains secretory piece (S) free secretory piece (s) and albumin (A) The I determinant characteristic of free secretory piece was accessible only in a minor proportion of native IgA molecules (in A) as demonstrated by faint precipitates in stained immunoplates (horizontally hatched areas above S and s) After reduction alkylation (in B) a substanti... tory piece (black columns above s) A mixtu... produced double precipitin rings with R 123

fraction A apparently lacked the A_1 determinant since it was antigenically deficient compared with native colostral IgA and free SP in reactions with R 77 Fraction B contained two molecular species, one was similar to native free SP whereas the other was antigenically deficient The latter lacked the I determinant since its precipitin line fused with that of secretory IgA in reactions with R 123 After 0 90 M βME only molecules deficient in both the I and A_1 determinants were detected in fraction B

Released and denatured SP was generally eluted like native free SP, but the SRID patterns indicated degradation and loss of reactive material—particularly after 0 90 M βME (Fig 9) Fragments with a size like immunoglobulin light chains and with the I and A_1 determinants largely inactive, were detected in pool C after extensive concen

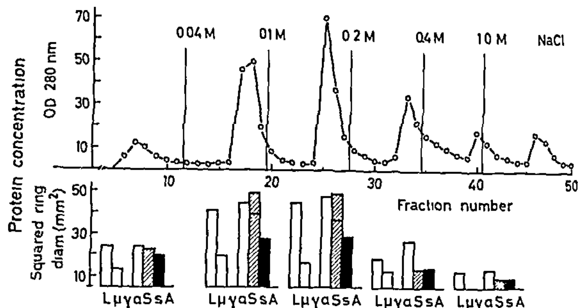


Fig 11B

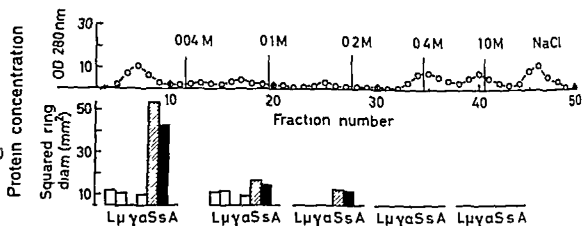


Fig 11C

tration (*Fig* 10B) Lack of *precipitation* could not be ascribed directly to molecular degradation however for SP fractions with denatured I determinants did not combine *with specific antibody in adsorption tests* (*Harboe et al* 1965)

The anionic exchange properties of released SP were compared with those of the native *free component which was eluted from* DEAE cellulose largely in the fall through fraction with 0 01 M phosphate at pH 7 5 (*Brandt aeg* 1971b) A 2 5 ml sample *of IgA* (*3 5 mg/ml*) *purified from colostrum* by gel filtration was reduced with 0 1 M βME and alkylated A native control sample was eluted mainly at 0 04 and 0 1 M NaCl (*Fig* 11A) The two *major fractions* contained respectively 1 per cent and 5 per cent contaminants—particularly IgM and lactoferrin The test sample exhibited a *fairly comparable protein elution pattern* and the two major fractions contained most of the released SP (Fig 11B) In contrast the released component obtained by gel filtra

tion of similarly reduced alkylated colostral IgA, was eluted from DEAE cellulose like native free SP (Fig 11C)

The possibility of studying degradation of secretory IgA by direct quantitation of released SP was considered A sample of colostral IgA was treated with 0 1 M βME and alkylated, another was reduced with 0 06 M cysteine The respective quantities of free SP, detected directly by SRID, were 15 per cent lower and 8 per cent higher than those estimated after gel filtration of the same samples The first estimates thus appeared reliable in view of the possible accumulation of errors during quantitation of individual chromatographic fractions Direct determination of released SP was consequently used to evaluate the effect of different types of reducing agents and alkylation on the degradation of secretory IgA However, the data presented below should not be taken as absolute figures When compared with native free SP in anti I immunoplates, released SP reacted more slowly and formed precipitin rings with more blurred edges— particularly after use of DTE This apparent antigenic difference between standard and test protein most likely influenced the quantitative results

Duplicate samples (200 μl) of colostral IgA (0 6 mg/ml) were reduced as described in Table 2 The yield of released SP increased about 110 per cent with βME and about 40 per cent with DTE following alkylation, whereas with cysteine there was no significant effect Alkylation was therefore omitted in other cysteine experiments, but was routinely used after reduction with βME and DTE The three reagents were compared over a great range of molarities on the basis of mean quantities of SP released in duplicate samples of colostral IgA (Fig 12) Maximum release was quite similar in the three systems, and occurred after 5 mM DTE 0 05–0 075 M cysteine, and 0 075 M βME Higher concentrations of the reagents resulted in decreased yield, apparently because of SP denaturation This was substantiated by tests with duplicate samples (200 μl) of colostral free SP, after treatment with βME

TABLE 2 *Effect of Alkylation on the Release of SP (mg/100 ml) from Colostral IgA (0 6 mg/ml) Reduced with Cysteine (Cys), β Mercaptoethanol (βME) or Dithioerythritol (DTE)*

Reagent	Molarity	Alkylation	SP
Cys	0 100	+	17 5
		—	19 5
βME	0 100	+	18 6
		—	8 7
DTE	0 005	+	19 9
		—	14 2

and DTE, even at extremely low molarities, the measurable amounts decreased drastically (Fig 12) Cysteine, however, had a small effect on both released and naturally free SP

The effect of cleavage of noncovalent bonds on the release of SP was studied by chromatography on Sephadex G 200 equilibrated with acid urea The separating properties of the gel column, and the possibility of testing the fractions by SRID without removing the urea, were examined with two 1 ml samples of colostrum containing bound and free SP Precipitation with antisera was not inhibited, and R 123 revealed the expected bimodal distribution of SP (Fig 13) A relatively low polymer peak indicated increased reactivity of bound SP due to molecular unfolding with exposure of I determinants A concomitant increase in polymer size (cf 0 90 M βME in Fig 9) could not be shown, however, for the exclusion limit of Sephadex G 200 under these conditions was about 200,000 (*Andreus* 1970) Free SP apparently exhibited smaller size and better reactivity in the sample with 15 mg of albumin marker—possibly because the high protein concentration stabilized the structure of the free component This was not further investigated, and the marker was omitted in subsequent denaturation experiments

The same column was used to examine the following 1 to 1 5 ml samples which had been dialyzed at 4° C for 18 hr against acid urea (Fig 14) *A* Fraction A from "Con

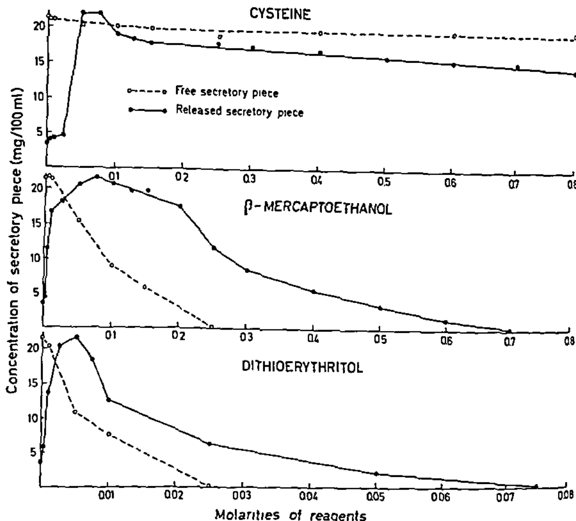


Fig 12 Effect of various reducing reagents on the release of secretory piece from colostral IgA and on the denaturation of free secretory piece Samples of colostral IgA (60 mg/100 ml), and samples of native free secretory piece (22 mg/100 ml), were treated with different molarities of cysteine β mercaptoethanol (followed by alkylation) or dithioerythritol (followed by alkylation) and the amounts of reactive secretory piece were quantitated by SRID with antiserum specific for the I determinant.

trol' in Fig 9 containing native secretory IgA, *B* Pooled fractions A from '0 015 M βME" and '0 09 M βME" in Fig 9, representing mildly reduced secretory IgA free from released SP *C* Pooled fractions 40–44 *from experiment described in Fig 8, representing* intermediately reduced secretory IgA free from released SP and *D* Pooled fractions A from '0 50 M βME' and "0 90 M βME" in Fig 9, representing strongly reduced secretory IgA free from released SP

Denaturation of native colostral IgA resulted in molecular unfolding (Fig 14A) As *mentioned above this was demonstrated by* a strong reaction of residual bound SP with antiserum specific for the I determinant There was only a minor release of SP and of 7S IgA The majority of these units are *thus disulfide linked in the secretory polymers*, and the elution pattern indicated that those released were in part SP-IgA complexes Because of molecular dissimilarities, the peaks of retarded components were overestimates *as compared with the polymer* peak This also applied to the released fraction of light chains whose elution position

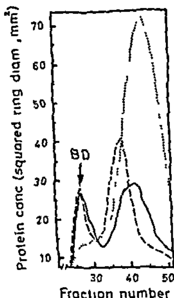


Fig 13 Bimodal distribution of secretory piece after chromatography of human colostrum on Sephadex G-200 equilibrated with 6M urea at pH 3 5 *Column size*, 2 5 × 36 *cm*, *flow rate* (upward), 1 ml cm⁻² hr⁻¹, fractions, 2 4 ml The samples which had been dialyzed against the urea solution, were 1 ml of colostrum (dashed line) and 1 ml of colostrum (solid line) containing 15 mg of albumin marker (dotted line) as well as 2 mg of blue dextran (BD) The elution patterns of secretory piece and albumin were determined by SRID with antiserum R 123 and R 128 respectively

was compatible with that of covalently stabilized dimers (*Grey et al* 1968) Their removal could not account for the exposure of I determinants in the bound SP, for the majority of light chains remained disulfide-linked in the polymer fraction

Denaturation of mildly and intermediately reduced colostral IgA likewise resulted in unfolded IgA polymers (Fig 14B, C) Their size appeared slightly smaller than that of the denatured, unreduced ones—probably because of a substantial removal of single light chains There was also release of α chains which were eluted like dimers, and the distribution of light chain determinants demonstrated a larger 7S IgA fraction than that obtained after denaturation of native polymers This verified that following moderate reduction some of the IgA is turned into noncovalently bonded dimers Such treatment had not raised the proportion of noncovalently linked SP, however, for the amount of this component released by denaturation was not increased Strongly reduced material was mainly split into monomers and heavy and light chains after denaturation (Fig 14D), but polymers containing SP still remained although exhibiting poor antigenicity Bound and released SP were virtually devoid of reactive I determinants

Precipitation of denatured colostral IgA by the adsorbed R-123 (Fig 4) could thus be ascribed to molecular unfolding rather than to release of SP The unfolding effect of different denaturing reagents was therefore evaluated directly by SRID with anti I (Fig 15) Disulfide exchange was blocked by iodoacetamide Urea alone (pH 7 5), or glycine-HCl buffer alone (pH 2 8), was not so effective as a combination of the two (pH 3 5), and the latter component was more denaturing than the former—although the difference varied between the IgA preparations tested Acetic acid at 0 1 M (pH 2 9) had little effect, whereas 1 M propionic or acetic acid (pH 2 4) appeared as effective as acid urea At pH 2 4 there was some nonspecific precipitation of antiserum proteins, but the specific reactions could be evaluated after washing and staining of the immunoplates Colostral IgA denatured by acid urea and dialyzed for 48 hr against PBS or 0 01 M phosphate buffer (pH 7 5), in the presence or absence of light chain dimers, was not refolded to such an extent that I determinants became masked

The effect of reduction and denaturation on the polymer conformation of secretory IgA was further examined immunochemically with R-54 made monospecific for the P determinant Controls of serum IgA polymers and dimers (P T) were included In double diffusion the antiserum produced a reaction of identity between colostral IgA and serum IgA polymers (*Brandtzaeg et al* 1970), and between serum polymers and dimers (P T), thus its activity apparently corresponded to the IgA dimer conformation

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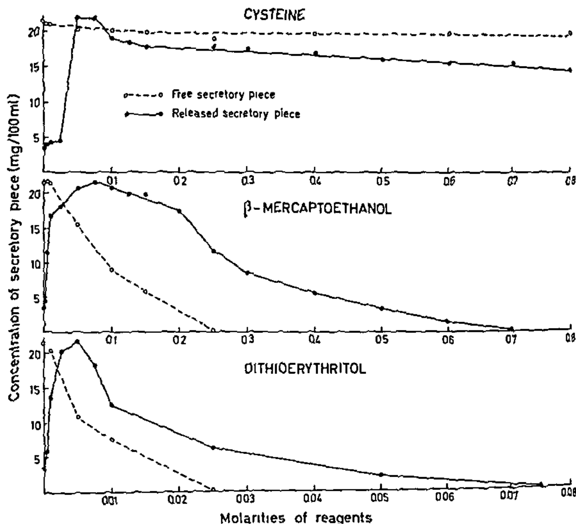


Fig 12 Effect of various reducing reagents on the release of secretory piece from colostral IgA and on the denaturation of free secretory piece Samples of colostral IgA (60 mg/100 ml), and samples of native free secretory piece (22 mg/100 ml) were treated with different molarities of cysteine β-mercaptoethanol (followed by alkylation) or dithioerythritol (followed by alkylation), and the amounts of reactive secretory piece were quantitated by SRID with antiserum specific for the 1 determinant.

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Denaturation of native colostral IgA resulted in molecular unfolding (Fig 14A) As mentioned above this was demonstrated by a strong reaction of residual bound SP with antiserum specific for the 1 determinant There was only a minor release of SP and of 7S IgA The majority of these units are thus disulfide linked in the secretory polymers, and the elution pattern indicated that those released were in part SP-IgA complexes Because of molecular dissimilarities the peaks of retarded components were overestimates as compared with the polymer peak This also applied to the released fraction of light chains whose elution position

which it was still loosely associated Similarly reduced alkylated SP separated from IgA by gel filtration, behaved like native free SP in anionic exchange (Fig 11C) and immunoelectrophoresis (*Brandtzaeg* 1971 b) The noncovalent interactions seem to depend on the integrity of SP, after use of βME above 0 15 M the growing proportion of molecules lacking I determinants exhibited increased immunoelectrophoretic mobility (Fig 5)

The major molecular species revealed by gel filtration of reduced alkylated colostral IgA are depicted to the right in Fig 17, and will be discussed in the order they are labeled

(*a*) Residual polymers containing SP largely bound by covalent as well as noncovalent linkages Following moderate reduction they are immunochemically similar to native secretory IgA, except that the reactivity of P determinants is greatly diminished above 0 1 M βME Compared with the same determinant in normal serum IgA polymers, however, that of secretory IgA appears slightly more resistant to reduction alkylation and definitely so to denaturation with acid urea This supports the view that SP stabilizes the quaternary structure of secretory IgA After high concentrations of βME, the A_1 determinant is destroyed and the polymers appear slightly unfolded with some accessibility of the I determinant Their amount decreases with increasing reductive conditions up to about 0 1 M βME, the remaining population seems to be extremely resistant and survives to a great extent as polymers even after 0 5–0 9 M βME followed by acid urea

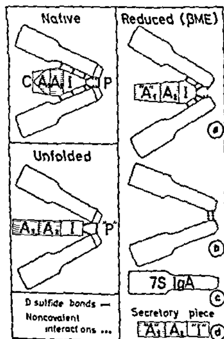


Fig 17 Diagrammatic representation of the effect of denaturation (molecular unfolding) and reduction (βME)-alkylation on the structure and antigenicity of human secretory IgA The following antigenic determinants are considered C, conformational characteristic of bound secretory piece A_1 and A_2 accessible determinants of bound and free secretory piece, *I*, determinant normally inaccessible in bound secretory piece, and P, polymer specificity of IgA dimers Determinants enclosed in quotation marks are liable to inactivation (cf text) The number of disulfide and noncovalent bonds drawn between molecule is arbitrary

(*b*) Noncovalently linked IgA dimers devoid of polymer specificity persist after major reductive removal of SP— that is, up to about 0 15 M βME

(*c*) IgA monomers are released to a rather small extent under mild reductive conditions, large amounts are liberated only following treatment that in addition affects noncovalent bonds (high concentrations of βME, or acid urea)

(*d*) Released SP occurs in detectable amounts even after extremely mild treatment—that is 5 mM βME or 0 5 mM DTE After 0 015 M βME—a concentration hardly affecting IgM—about 80 per cent of the obtainable reactive SP is liberated It is therefore wrong to state without reservation that secretory IgA appears "quite resistant to reductive cleavage' (*Tomasi* & *Bienenstock* 1968) High resistance is characteristic of only a minor polymer fraction, and the total population appears heterogeneous with respect to the covalent bonding of SP

In this study "maximum yield' of released SP refers to molecules appearing immunochemically similar to *native free* SP in double diffusion tests Even these molecules

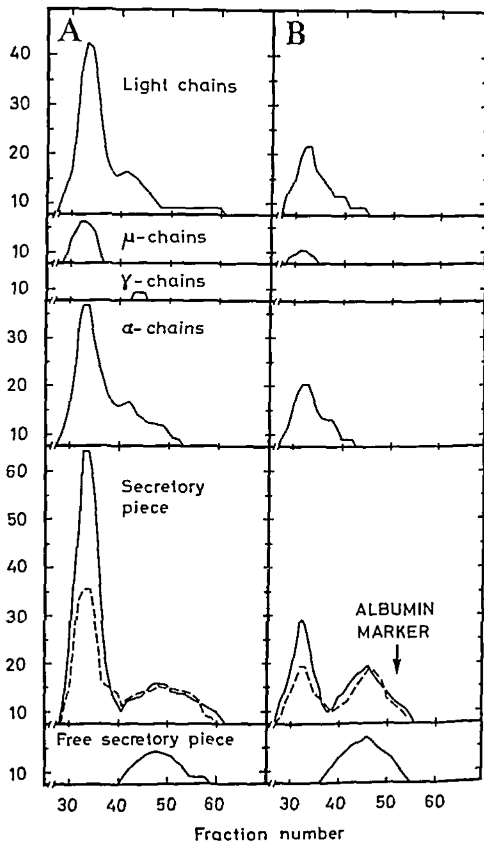

A
B
Light chains
μ-chains
γ-chains
α-chains
Secretory piece
Free secretory piece
ALBUMIN MARKER
Protein concentration (squared ring diameter, mm²)
Fraction number
10
20
30
40
50
60

Fig 2.

tography Gel filtration was also inefficient for separation between IgG and SP, whereas polymers and monomers of IgA in the main could be avoided The optical density of the first DEAE cellulose fraction (Fig 4D) indicated however, that the obtained SP was not as pure as cSP 1 (Fig 4B), in addition to the same contaminants (about 12 per cent) as those in the colostral preparation, it contained about 20 per cent of amylase (Fig 6) Purified parotid SP migrated considerably faster than native parotid IgA in immunoelectrophoresis (Fig 6)

The samples of free SP were compared in immunoplates containing antiserum monospecific for the I determinant Preparation cSP 1 was considered the best primary standard and its concentration was taken to be total protein (Biuret) minus detected contaminants The concentration of cSP 2 estimated with reference to cSP-1 agreed well with the Biuret-determined value minus contaminants, whereas the immunochemical estimate of cSP 3

Fig 2 Elution patterns of immunoglobulin components of colostrum after chromatography on Sephadex G 200 Column size 2 5 × 37 cm, flow rate 2 2 ml cm⁻² hr⁻¹, fractions 2 4 ml Samples A 0 5 ml colostrum containing 14 0 mg IgA and 1 0 mg free SP per ml B 0 5 ml colostrum containing 6 7 mg IgA and 1 8 mg free SP per ml Arrow indicates elution position of the albumin marker added to one of the samples Distribution of immunoglobulin components was determined by SRID with antisera specific for light or heavy chain determinants Total (free and bound) SP was registered with a serum (R 123) from a rabbit immunized with free SP (solid line) as *well as* with a serum (R 77) from a rabbit immunized with bound SP (dashed line) Free SP alone *was* registered with antiserum R 123 made *monospecific* for the I determinant The squared diameters of the precipitin rings were used as *relative* concentration estimates

Fig 3 Elution patterns of immunoglobulin components and lactoferrin in a preparation of partially purified parotid free SP (2 7 mg) after chromatography on Sephadex G 200 *Conditions and registration* methods were as described in Fig 2 Arrow indicates elution position of blue dextran (BD) marker (void volume) *Lactoferrin was registered* with antiserum R 125 at a final dilution of 1 10 Fractions were pooled as indicated (hatched area), precipitated at 70 per cent saturation *with* ammonium sulfate, and further purified by anionic-exchange chromatography (cf Fig 4D)

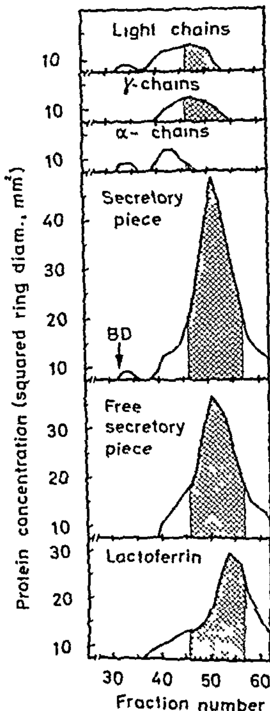


Fig 3

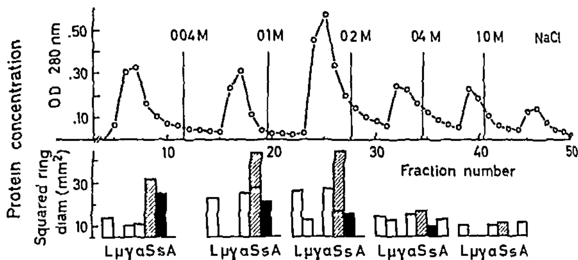


Fig 4A

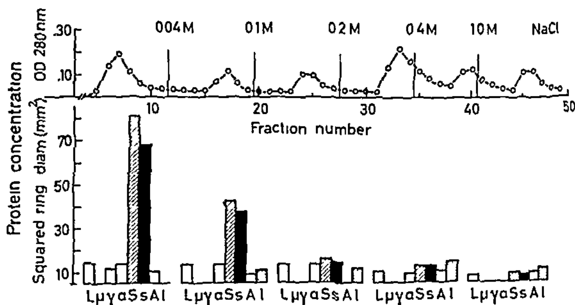


Fig 4B

Fig 4 (A, B, C and D) DEAE-cellulose chromatography of whole colostrum, fractions of colostrum, and partially purified parotid free SP Column size, 1 4 × 8 cm flow rate, 40 ml cm^{-2} hr^{-1}, fractions, 2 4 ml Samples were in A, colostrum (1 8 ml), in B, free colostral SP (2 6 mg) partially purified by Sephadex G-200 filtration and ammonium-sulfate precipitation (4 4 ml OD $_{280\,nm}$ ≈ 1 3) in C supernatant fluid separated from sample B by ammonium-sulfate precipitation (3 5 ml, OD $_{280\,nm}$ = 5 5), and in D, free parotid SP (1.7 mg) partially purified by anionic-exchange and Sephadex chromatography (cf Fig 3) and repeated precipitation with ammonium sulfate (4 1 ml, OD $_{280\,nm}$ = 1 3) Proteins were

Fig 4C

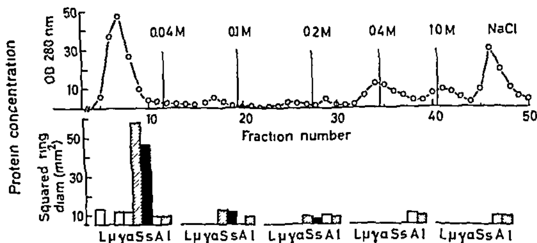


Fig 4D

eluted stepwise with 0 01 M phosphate buffer pH 7 5 containing increasing molarities of NaCl as indicated *Above* Optical density (OD) of fractions *Below* Protein composition of the fraction with maximum OD for each buffer step (except 1 0 M NaCl) as revealed by SRID with antisera to light (L) or heavy (μ γ and α) immunoglobulin chains secretory piece (S), free secretory piece (s), albumin (A) and lactoferrin (l) A mixture of secretory IgA (bound secretory piece) and free secretory piece produced double precipitin rings with R 123 (indicated by horizontal line in columns above S, cf also Fig 8) Anti lactoferrin (R 125) was used at a final dilution of 1 2 (not 1 10 as in *Fig* 3)

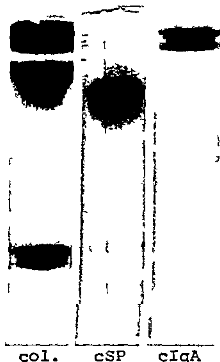
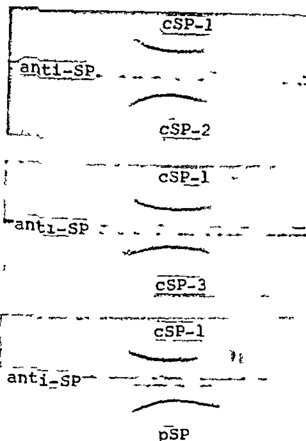


Fig 5 Disc electrophores s of 10 μl colo strum (col) 60 μg of a preparation containing 89 per cent of free SP (cSP) and 25 μg of purified colostral IgA (cIgA) Anode at bottom



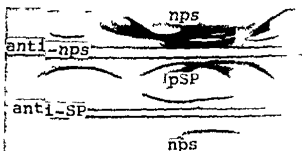


Fig 6 Colostral (cSP) and parotid (pSP) free SP and normal parotid secretion (nps) tested by immunoelectrophoresis against *antiserum to SP (anti SP) and to parotid* secretion (anti nps) Parotid SP contained albumin and amylase as major contaminants Free SP in whole parotid secretion was precipitated along with bound SP *(secretory IgA) in a cathodally located* line Anode to the left

TABLE 1 *Content of Free SP (per Cent in Colostral Preparations*

Preparation	Total protein (Biuret	Free SP A	Free SP B	(A B 100 / A
cSP 1	100	89		
cSP 2	100	68	62 7	8
cSP 3	100	97 5	50 3	48

A Total protein m nus contam nants
B Determined immunochemically with reference to the value A of preparation cSP 1

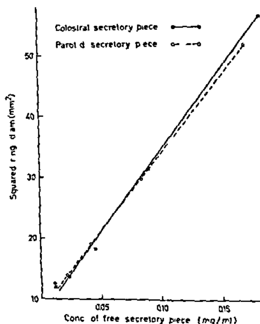


Fig 7 Standard curves for the quantitation of free secretory piece by single radial immunodiffusion The concentration of the colostral standard preparation (cSP 1) was determined as value A in Table 1 that of the parotid preparation was estimated immunochemically with reference to the colostral standard

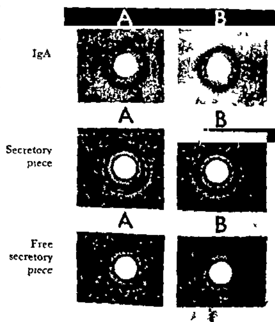
was 48 per cent too low (Table 1) The content of free SP in the parotid preparation was also determined immunochemically with reference to cSP 1 Standard curves based on dilutions of these two preparations were almost identical (Fig 7) and the slight difference between the slopes might be ascribed to a systematical dilution error

Fig 8 Single radial immunodiffusion of colostrum in immunoplates specific for IgA (anti-α chain) for secretory piece (R 123), and for free secretory piece (anti I-determinant) Samples A containing 24 5 mg IgA and 4 2 mg free secretory piece per 100 ml and B containing 17 7 mg IgA and 5 0 mg free secretory piece per 100 ml Note that R 123 produces a double precipitin ring when the ratio of free to bound secretory piece is small and that the size of the precipitate produced by anti I appears unrelated to the concentration of bound secretory piece (secretory IgA)

Quantitation of Free SP

The determinations were based on a working standard of free SP calibrated with the colostral preparation cSP-1 (cf above) By testing different samples of colostrum, the concentrations obtained for free SP appeared unrelated to those of secretory IgA with bound SP (Fig 8) Tests of gel filtration fractions (cf Fig 2) confirmed the apparently specific precipitation of free SP by the anti serum used (anti I), although stained immunoplates revealed a faint reactivity of the polymer fraction (cf *Brandtzaeg* 1971) The relationship between free SP in two samples of colostrum (Fig 2) was virtually the same (5 per cent difference), however, when estimated before and after gel filtration The influence of a highly varying amount of bound SP on the quantitation of free SP was therefore considered negligible

Free SP was present in all of the normal parotid secretions tested (Table 2), its amount was below the level detectable by direct quantitation in only one sample (S G), but was revealed after concentration The level of free



some and inaccurate, and no definite quantitative data were obtained for free SP

The second problem mentioned above has not as yet been solved although it should be possible with more advanced techniques Free SP exhibits polydispersity in size and charge (Figs 2, 4 and 6), and also upon salt fractionation These properties render difficult the isolation of a representative standard protein by conventional biochemical methods Amylase was a major contaminant of the parotid preparation Better purity could probably have been obtained by Sephadex G-75 chromatography, but previous experiments (*Brandtzaeg et al* 1970) indicated incomplete separation between the enzyme and SP Lactoferrin was the major contaminant of the colostral preparation It could be removed by ammonium-sulfate precipitation at 55 per cent saturation (*Newcomb et al* 1968), but this resulted in a substantial loss (about 60 per cent) of free SP For both the parotid and the colostral preparation a narrow sampling of Sephadex G 200 fractions was necessary to avoid contamination with IgG and albumin, this interfered with the polydispersity of the standard protein It has been recommended to use external secretions from patients lacking IgA as a convenient starting material for the isolation of free SP (*Tomasi* 1970) The problem with the major contaminants, however, will be the same as above Such fluids moreover are not so superior in content of free SP to normal secretions as previously assumed (cf below)

Best purity was obtained by isolating SP from reduced alkylated colostral IgA and this has been the method of choice up to now (*Tomasi & Bienenstock* 1968) However, despite much milder conditions than those recommended by others (*Tomasi & Bienenstock* 1968), the preparation was highly inactivated with regard to I determinants (Table 1) This agreed with the previously demonstrated susceptibility to reduction alkylation exhibited by the I and the A_1 determinant of SP (*Brandtzaeg* 1968, 1971) Even after the use of still milder conditions, the immunochemical identity of released and native free SP is questionable (*Brandtzaeg* 1971) In conclusion, therefore, colostrum or milk (*Munster et al* 1968) seems to be the best starting material for the isolation of a standard preparation of free SP Specific immunosorbent techniques would be preferable to avoid contamination and at the same time obtain a protein with representative polydispersity

Appreciable polydispersity is characteristic of epithelial glycoproteins (*Gibbons* 1966) and could thus be expected for free SP Its relatively high content of glycine and carbohydrate (*Tomasi* 1970) also agrees with features common to proteins in this group (*Gottschalk* 1966) Their basic structure is that of a polypeptide to which carbohydrate chains in varying numbers are linked covalently (*Gottschalk* 1966) The high susceptibility of the I and A_1 determinants of SP to reduction alkylation (cf above) may thus indicate that the carbohydrates are involved in their conformation It is furthermore possible that the carbohydrates participate in the specific conjugation of SP to IgA

The atypical behaviour of free SP upon acidification (Fig 1) could probably also be ascribed to its content of carbohydrate, for the precipitation pattern was similarly exhibited by blood group substances but not by proteins with a smaller proportion of carbohydrate such as the immunoglobulins *Goa* (1953) included the acid precipitation in Biuret determinations to avoid non protein contaminants capable of giving a color reaction (cf *Piper et al* 1965) He used trichloroacetic acid which, however leaves several glycoproteins soluble (*Daues* 1965), and his method has subsequently been modified (cf Materials and Methods) The Biuret reaction is considered the most reliable method to obtain concentrations in good correspondence with the amount of polypeptide present (*Peters* 1968), particularly for samples containing glycoproteins it appears superior to other tests, e g, the Folin Ciocalteu or Lowry method (*Mandel et al* 1965) But with the horse serum standard used in the present study the estimate of SP could at best refer

to its actual polypeptid moiety and not to its total concentration Since no exact knowledge about the proportion of carbohydrate in SP is available, a correction for this moiety can not presently be made Dry weight would thus be the most fundamental concentration estimate (*Peters* 1968) of a primary SP standard, but this requires a larger quantity of purified material than that available in our laboratory

In view of the many problems involved in obtaining a representative and defined primary standard of free SP, the colostral preparation cSP-1 used in the present study should be considered provisional The quantitative data presented for free SP in biological fluids are consequently approximate values, although comparisons made between various types of secretions may be valid There was thus no significant difference between the slopes of the colostral and parotid standard curves (Fig 7), and previous studies (*Brandtzaeg* 1970a, 1971) have indicated similar immunochemical and physicochemical properties for free SP from different secretions, with the exception of considerable variations in mobility In immunoelectrophoretic tests of whole secretions, parotid free SP has thus been found to migrate much more slowly than its colostral counterpart (*Hanson & Johansson* 1967, *Brandtzaeg et al* 1970) This difference was almost eliminated, however, when parotid free SP was partly purified (Fig 6), its reduced mobility in whole secretions is therefore most likely due to interfering cathodally migrating substances particularly amylase

A striking observation was that appreciable concentrations of free SP were present in all of the tested samples of normal salivary fluids This was in contrast to the previously reported lack of free SP in saliva from most normal adults (*South et al* 1966 *Tomasi & Bienenstock* 1968) but agreed with our studies of pooled secretions (*Brandtzaeg et al* 1968 1970) Earlier investigators who used double diffusion tests noted the regular presence of free SP only in saliva from young children whose IgA had not reached adult levels (*South et al* 1968) The reason was that with the conventional antisera the precipitin lines of free SP and secretory IgA are prone to be superimposed unless the concentration of the latter component is relatively low (cf Fig 9) By chromatography, however, free SP has previously been found in colostrum (*Newcomb et al* 1968), milk (*van Munster et al* 1969) and urine (*Bienenstock & Tomasi* 1968) from normal adults, and this study demonstrates its presence also in normal samples of nasal fluid and sweat

The various types of secretions showed an interesting relationship by exhibiting SP to IgA ratios within highly overlapping ranges With regard to these two secretory proteins the external fluids tested could thus be characterized as quite similar except for their extremely different dilution factors There was a trend, however, for the samples with the highest concentrations of IgA to exhibit

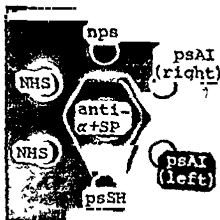


Fig 9 Detection of free secretory piece by the conventional method with antiserum to secretory IgA [anti (α chain + SP)] in double diffusion Samples nps pooled normal parotid secretion (SP IgA = 0 3), psAI parotid secretion from the right (SP IgA = 0 4) and the left (SP IgA = 0 7) gland from the same individual and psSH parotid secretion (deficient in IgA) from a hypo-γ globulinemic patient The ratio of free SP to secretory IgA (SP IgA) varied as indicated above Note that when this ratio is low, it is difficult to detect free SP because its precipitate is included in that of secretory IgA

The elimination of Kanamycin from the foetus is slow Detectable concentrations persist in the foetal blood serum and in the amniotic fluid roughly for at least 48 and 72 hours, respectively, following cessation of the medication to the mother

Our observation that a build up occurs in the amnion, to a level surpassing that of the foetal serum, and that this build up continues even after cessation of medication to the mother indicates that the foetal kidney is taking some part in the handling of the Kanamycin in the foetus, in quantitative terms however, this mechanism is only secondary

Our findings are in good agreement with experiments carried out by *Mascherpa et al* (1966) who administered single doses of Streptomycin, Cephalosporin and Rifamycin to rabbits Their investigations revealed prolonged retention in the foetus as compared with the maternal animal retention being most prolonged in the amniotic fluid

Falbe-Hansen (1967), who applied Kanamycin to pregnant rats, found degeneration of the organ of Corti in the mother animals, but not in the offspring

Shida et al (1967) injected Kanamycin into pregnant Guinea pigs and found degeneration of the organ of Corti in the mother, but not in the foetus

Our results (a o that the foetal serum concentration only reaches a level of about 7 per cent of that of the maternal serum) seem to provide the basic explanation of, and to support, the toxicological investigations by *Falbe Hansen* (1967) and *Shida & al* (1967)

The discrepancy between these studies and those published by *Kreis* (1966), however seems still open to discussion

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The Kanamycin used in the study was kindly placed at our disposal by the courtesy of Messrs. *H Lundbeck & Co*, who also carried out the bioassays

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Acta path microbiol scand Section B 79 209–218, 1971

THE PYROGENIC RESPONSE TO ENDOTOXIN IN WARFARIN-TREATED RABBITS

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No difference was found between warfarin treated and untreated rabbits with regard to the pyrogenic response to *Salmonella typhi* endotoxin by comparison of the contours of the fever curves the fever indices, the latent periods and the various temperatures (1 hr 3 hr and maximum temperature) A constant low coagulation activity is therefore of no consequence to the pyrogenic reaction provided the endotoxin is intravenously administered This strongly indicates that the endotoxin induced alterations of the blood coagulability have no decisive influence on the pyrogenic response The dose response relationship was more reliably determined in tolerant than in nontolerant rabbits The fever index seemed to be the most reliable of the response parameters in discriminating between doses The relation between the blood coagulability and the R E S function is discussed

Administration of endotoxin profoundly alters the function of the reticuloendothelial system (R E S) as measured by the phagocytic clearance capacity The first endotoxin injections provoke a hypofunction of short duration, followed by a hyperfunction A more constant hyperfunctional state gradually develops on further injections (7, 9, 20, 23, 31, 35) The functional state of the R E S greatly influences the endotoxin induced pyrogenic response including the development of the pyrogenic tolerance (5 13–15, 31) However, recent observations may indicate that antibody formation and cellular desensitization are the most important determinants of development of tolerance (15 30)

Concurrently with the changes in the R.E.S function induced by the endotoxin alterations of the blood coagulability take place (23, 28) Similarly, the first injections result in phases of hyper- and hypocoagulability, further injections leading to a gradual depletion of fibrinogen, platelets and clotting factors (10, 12, 22, 23, 25, 38) By some unknown mechanism the endotoxin seems to trigger an intravascular coagulation by conversion of fibrinogen to fibrin aggregates (12, 23, 28)

Several observations seem to indicate not only a time relation but also an interaction between the functional state of the R E S and the coagulability of blood Both fibrin aggregates and endotoxin are simultaneously phagocytized by the R E S (23, 28) Blockade of the R.E S function alters the coagulation system (23, 29, 38), and anticoagulant treatment lowers the clearance rate of particles (1, 16) Moreover, the origin of some clotting factors may be the Kupffer cells of the R E S (12)

Received 28 viii70

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TABLE 2 *The Mean and Standard Error (S E) for the Latent Periods, Fever Indices, and the 1hr, 3 hr and Maximum Temperature Rise of Each of Four Groups of Rabbits after the 1st and 16th Injection of Endotoxin (L P) from S typhi 0901*

L P mcg/kg b w	Latent period min		Temperature rise (Δ T) °C						Fever index (F I) cm²	
			1 hr		3 hr		Max			
	Mean	S E	Mean	S E	Mean	S E	Mean	S E	Mean	S E
					NONTOLERANT*					
0 04	23 9	2 7	1 1	0 12	1 8	0 09	2 0	0 13	123 9	9 2
0 2	15 4	3 1	1 3	0 23	1 5	0 28	1 7	0 22	108 8	20 2
1 0	22 3	5 8	1 4	0 23	2 2	0 22	2 3	0 29	156 6	21 9
5 0	14 1	1 6	1 2	0 14	1 5	0 43	1 7	0 30	119 1	26 3
					TOLERANT§					
0 04	34 8	4 3	0 8	0 15	0 4	0 06	0 8	0 14	34 6	4 4
0 2	27 4	5 3	0 8	0 30	0 4	0 11	1 2	0 24	39 7	4 7
1 0	23 0	1 8	1 4	0 13	1 1	0 43	1 7	0 18	69 0	13 7
5 0	18 0	3 0	1 4	0 74	1 1	0 09	1 7	0 29	81 6	10 8

* Nontolerant After the 1st injection
§ Tolerant After the 16th injection

The mean and S E for the fever indices, the latent periods, and the 1-hr, 3 hr and maximum temperatures of each group for nontolerant (1st injection) and tolerant (16th injection) rabbits are given in Table 2 and the mean values are visualised in Fig 2 A dose response relationship similar to that indicated for the 16th injection was found in tolerant rabbits for each of the other injections When the median values of the different response parameters are calculated for each group, these values except for the latent period, do not diverge very much from t[illegible] mean values

Expressed by the F I, and the 1 hr, 3 and maximum temperatures, the dose resp se relationship for the 1st injection (nonto rant), diverges from the expected rela ship and from that for tolerant rabbits (2) In tolerant rabbits the responses parallel to the increase of dose, the d response curves being steepest for the of doses in the dose range *0 2–1 0* m kg b w Correspondingly, with the

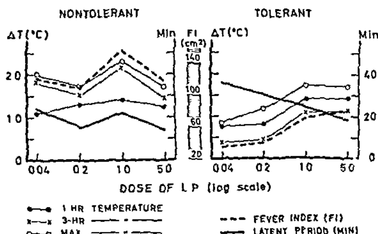


Fig 2 The dose response of the response measured index, latent period, and 3 hr and maximum temp rise, to endotoxin (L P) *typhi 0901* in nontolerant lerant rabbits

of dose from 0 2 to 1 0 mcg/kg b w , the val ues of each response parameter, except the 3-hr temperature increase significantly The high S E. of the 3 hr temperature for the dose of 1 0 mcg/kg b w is noteworthy In nontolerant rabbits the increase of dose from 0.2 to 1 0 mcg/kg b w gives an increase of the responses which is significant when meas ured by the F I and the 3 hr temperature, and almost significant when measured by the maximum temperature (0 10>P>0 05) However although there is no significant difference between the responses to 0 04 and 0 2 mcg/kg b w the increase of the respon ses with increase of the dose from 0 04 to 1 0 mcg/kg b w is not significant for any para meter at the 0 05 per cent level This discre [illegible] in nontolerant rabbits between the re sponse to the lowest doses is later discussed

The latent period (Fig 2) seems to de crease linearly on increase of the log dose in [illegible] and tolerant rabbits this being [illegible] marked when the median values [illegible] The test of linearity gives no reason to [illegible] the hypothesis of linearity, and re gression analysis shows the decrease to be [illegible] for both nontolerant and tolerant rabbits. It should however be noted that the latent period is a compound unit of measure representing both the real latent period and the [illegible] of temperature increase

The [illegible] C V of the different re sponse parameters, including the latent pe riod, for the different groups is in general lowest for the F I followed by the C V for the latent period The C V for the maximum temperature is somewhat more homogeneous and, on the average, lower than those for the 1 hr and 3 hr temperatures

It may be concluded that in this experi ment the best reflection of the dose response relationship was found in tolerant rabbits Differences in the responses are best revealed in the log of doses in the dose range 0 2–1 0 mcg/kg b w Although none of the response parameters is particularly prominent in dis criminating between doses the F I seems to be the most reliable

Pyrogenic Response and Tolerance in Warfarin Treated Rabbits

The rabbits of groups I A and I were chosen for inoculation of 0 2 mcg L P /kg b w daily and the rabbits of groups II A and II for 1 0 mcg L P /kg b w for a test period of 16 days The rabbits of groups I A and II A were in addition treated with warfarin The recipients of equal doses were experi mented on in succession taking two by two One rabbit was randomly chosen from each of the two different groups

The TT c_t V P C, E S R and rectal tem peratur of all rabbits were normal prior to

[illegible] *The Mean and Standard Error (S E) for the Fever Indices (F I) of the Rabbits of the [illegible] (I A II A) and the Untreated (I II) Groups after Daily Injection of Endotoxin (L P) from S typhi 0901*

0 2 mcg L P /kg b w				1 0 mcg L P /kg b w			
I		I A		II		II A	
F I		F I		F I		F I	
Mean	S E	Mean	S E	Mean	S E	Mean	S E
108 8	20 2	111 5	12	156 6	21 9	139 2	31 5
64	10 9	64 8	11 6	122 6	24 5	108 8	27 0
55 8	7 8	68 0	10 1	59 5	9 9	62 3	7 1
38 0	7 0	71 0	7 5	65 0	9 5	65 8	7 6
43 7	4 1	60 1	10 6	53 3	7 7	48 4	7 5
41 5	5 9	57 4	4 9	69 5	13 5	51 7	9 3
39 7	3 5	41 6	5 1	51 4	9 9	51 1	3 9
39 7	4 7	67 6	24 1	69 0	13 7	50 4	4 0

DISCUSSION

The methods reported for the measurement of a provoked pyrogenic response in rabbits vary considerably (8, 11, 21, 37)

In the present experiments the rabbits of each group were given a constant endotoxin dose per kg b w according to *Keene et al* (21) This method results in the endotoxin concentration in the plasma probably the determining factor of the pyrogenic response, being almost the same in the different rabbits, provided the V P C are similar An attempt was made to reduce variations in pyrogenic response by selecting young adult rabbits of about the same weight (21, 34) and with E S R values within normal range Further, all rabbits had rectal temperatures within normal range prior to the test and the mean temperature of each group was the same The temperature lability of each rabbit was therefore not tested as recommended by *Martin & Marcus* (24) A sex selection as recommended by others (21 34) was not made since the rabbits were examined separately and since no reports of a different pyrogenic response for the two sexes had been seen The most variable individual factor of the response probably lies in the conduct of the experiments especially in the temperature recording technique An undisturbed continuous recording as performed in the present work, is no doubt the most reliable method Furthermore it reflects the total fever curve which gives an impression of the whole pyrogenic response and permits the measurement of several response parameters

During the test period six rabbits died three having received the highest endotoxin dose and none the lowest The reason was probably a toxic effect as no other cause was to be found

In the present experiments a reliable distinction was obtained between the pyrogenic responses to the different doses in tolerant rabbits but not in nontolerant, or, at least not in connection with the first endotoxin injection (Fig 2 Table 2) The latter may be due to a disproportionately high fever response to the lowest dose This cannot be explained by an error of dosage since only one stock solution of each dose was used throughout More probably the rabbits have less ability to discriminate between doses on the first injection Irrespective of selection rabbits are temperature labile (24), and are probably more influenced by stress in connection with the 1st injection than with the subsequent injections Further, the experiments show that in the pyrogenic response of nontolerant rabbits the second temperature rise is the dominant one whereas the first rise corresponds to the one temperature rise seen in tolerant rabbits (Fig 2) Others have observed (2, 27) that the biphasic temperature rise is provoked by a first and a second release of endogenous pyrogen The present findings may indicate that the first amount of endogenous pyrogen released corresponds more closely to the amount of endotoxin administrated than the second amount of pyrogen released

The present results also indicate that in tolerant rabbits the 3 hr temperature gives a better reflection of differences in dose than the 1 hr and maximum temperatures With regard to the C V the F I seemed to give the most reliable indication of the dose response relationship in accordance with the findings on single endotoxin injections (21) The linear decrease of the latent period in tolerant rabbits after increase of the log dose is also in accordance with observations following single injections (2 11)

From the dose response relationship obtained including the dose dependant tolerance development it is seen that differences in the responses are best revealed for the log of doses in the dose range 0 2 1 0 mcg/kg b w Further increase of the dose to 5 0 mcg/kg b w gives high lethality and no further increase of the pyrogenic response Thus the maximum responses occur on doses in the dose range 0 2–5 0 mcg/kg b w most probably on doses of about 1 0 mcg/kg b w The two doses 0 2 and 1 0 mcg/kg b w should therefore give a good representation of the pyrogenic effect of endotoxin and be suitable

for the study of warfarin influence on the pyrogenic response

The present results seem to give a clear-cut answer to the main question of this work Warfarin treatment does not influence the pyrogenic response or the development of pyrogenic tolerance induced by endotoxin as determined by the contours of the fever curves (Fig 3 and 4), the F I, the latent period and the 1-hr, 3-hr and maximum temperature At least, this is valid when the coagulation activity is kept at the level corresponding to the therapeutic level in man (19) and the endotoxin is intravenously injected

The low coagulation activity resulting from warfarin treatment does therefore not influence the amount of endogenous pyrogen released, either on the first or the second release, as determined by a pyrogenic test (2, 6, 8, 27) The endogenous pyrogen is released from the polymorphonuclear leucocytes (2, 3, 6, 8), and the present results may therefore indicate that a low coagulability does not interfere with this mechanism

According to the present findings, it is not likely that the pyrogenic response is influenced by the alterations of coagulability following endotoxin mentioned in the introduction to this work (23, 38)

Owing to the relation between the R E S function and the pyrogenic response (5, 14, 15, 31), this assumption implies that the alterations in coagulability have no causal influence on the clearance rate of endotoxin This conclusion is, however, in contrast to the assumed connection between coagulability and the functional state of the R E S Should such a connection nevertheless exist, and this is strongly indicated by the earlier mentioned observations the present findings support the view that the R E S function, as measured by the clearance rate, has no decisive influence on the pyrogenic response (15, 30) Further investigations, especially on the relation between coagulability and the R E S function, are needed to clarify these problems

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Acta path microbiol scand Section B 79, 226–232, 1971

IMMUNOCHEMICAL INVESTIGATIONS ON *NEISSERIA GONORRHOEAE* ENDOTOXIN

1 *Characterization of Phenol-Water Extracted Endotoxin and Comparison with Aqueous Ether Preparations*

J A Mæland and T Kristoffersen

The University of Bergen, Scools of Medicine and Dentistry, The Gade Institute, Department of Microbiology and Laboratory of Oral Microbiology, Bergen, Norway

Phenol water extracted endotoxin from three strains of *Neisseria gonorrhoeae* were composed mainly of carbohydrate and lipid and contained much less protein than aqueous ether extracted endotoxin from the same strain All phenol water preparations contained glucose galactose glucosamine and heptose, i e the same sugars as those found in aqueous ether preparations In addition 2 keto 3 deoxy octulosonic acid (KDO) was demonstrated in the phenol water preparations Haemagglutination inhibition showed that, like aqueous ether preparations all phenol water extracted endotoxins contained an antigenic determinant probably of polysaccharide nature (determinant *a*) With respect to this determinant, they tended to exhibit a higher degree of serological activity than the corresponding aqueous ether preparations An antigenic determinant probably of protein nature (determinant *b*) found in aqueous ether preparations from all three strains was present in phenol water preparations from only two of the three gonococcal strains

Endotoxic materials prepared from whole cells of one strain of *Neisseria gonorrhoeae* (strain 8551) by extraction with aqueous ether, alkali trichloroacetic acid or by heating have been shown to contain at least two antigenic determinants as revealed by haemagglutination and haemagglutination inhibition techniques (19) One determinant, called determinant *a*, was found to be of carbohydrate nature and the other determinant *b*, of protein nature (19) Further experiments with aqueous ether prep ations provided evidence that both determin vere constituents of the endotoxin complex (20) Phenol water extracted endotoxin from the same strain of gonococci was found to contain only determinant *a* (19)

Aqueous ether extracted endotoxin contained large amounts of protein and only small amounts of carbohydrate and lipid (21, 23) By contrast, the phenol water preparations from gonococci examined by *Tauber & Garson* (30), contained mainly carbohydrate and lipid and only a minor protein or peptide component Some chemical and serological properties of phenol-water extracted endotoxin from strain *8551* and two additional strains of gonococci and a comparison with corresponding aqueous ether preparations are reported in the present paper

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MATERIALS AND METHODS

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Strains 8551, V and VII have been described in a previous report (23)

Cultivation and Harvesting

The bacteria were cultured on placental broth agar, harvested in saline, washed with saline and stored frozen until use

Preparation of Endotoxin

Extraction and purification of phenol water and aqueous ether extracted endotoxin were performed as previously reported (19, 20) Briefly, phenol water extracted endotoxin was prepared from whole bacteria by treatment with equal volumes of 90 per cent phenol and water at 37° C The endotoxin was recovered from the water phase by precipitation with acetone and washed with distilled water Aqueous ether preparations were obtained by extraction of bacteria with one volume of saline and two volumes of diethylether at 4° C All preparations were purified by treatment with DNase (DNase 1, B Grade, Calbiochem) and repeated washings with distilled water The purified preparations were lyophilized

Paper Chromatography

Samples were hydrolyzed in sealed tubes at 100° C with 3 N HCl for 3 hrs and with 0 1 N H_2SO_4 for 10 mins 1 N H_2SO_4 for 16 hrs or with 2 N H_2SO_4 for 3 hrs Acid was removed from the HCl hydrolysates by evaporation over NaOH pellets The H_2SO_4 hydrolysates were neutralized and prepared for chromatography as described by *Kauffmann et al* (13) Circular paper chromatograms using Whatman no 1 paper, were run with *n* butanol pyridine water (6 4 3) or ethyl acetate pyridine water (12 5 4) The dried chromatograms were sprayed with aniline hydrogen phthalate for the detection of aldoses alkaline silver nitrate for reducing sugars, the Elson Morgan reagents as recommended by *Smith* (28) for amino sugars or with the thiobarbituric acid reagent of *Warren* (32) for deoxy sugars

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Nitrogen was determined by the micro-Kjeldahl technique as described by *Kabat & Mayer* (12 Samples were digested for 8 hrs Protein was determined by the method of *Lowry et al* (15) with bovine serum albumin as standard Determination of total phosphorus was performed according to *Fiske & Subbarow* (4) with some of the modifications of *Youngburg & Youngburg* (36) Neutral sugars were measured by the orcinol method (34) with glucose galactose (1 1) as standard 2 Keto-3 deoxy-octulosonic acid (KDO) was measured by the method of *Weissbach & Hurwitz* (33) in samples hydrolyzed in 0 02 N H_2SO_4 for 20 mins at 100° C (25) using a standard prepared from a sample of 2,4,5,7,8, penta O acetyl KDO methyl ester (kindly provided by *Dr Eduard C Heath*, Baltimore, Md) by the method of *Ghalambor, Levine & Heath* (6) Hexosamines were measured as glucosamine HCl by the *Rondle & Morgan* method (27) Fatty acid esters were estimated as tripalmitin by the method of *Snyder & Stephens* (29) The presence of heptoses was investigated by the cysteine sulfuric acid reaction of *Dische* (3)

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Rabbit antisera to whole gonococci were the same as those used in previous studies (18 23)

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Sensitization of sheep erythrocytes with each of the determinants *a* and *b* was performed as previously reported (22) Endotoxin treated with alkali (0 04 N NaOH at 37° C, 18 hrs) was digested with pronase (B grade, Calbiochem) to obtain preparations which sensitized erythrocytes with determinant *a* The preparation sensitizing erythrocytes with determinant *b* was obtained by oxidation of alkali treated endotoxin (0 008 N NaOH at 37° C 18 hrs) with periodate (0 02 N sodium *meta* periodate, 20 hrs, 20° C)

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IMMUNOCHEMICAL INVESTIGATIONS ON *NEISSERIA GONORRHOEAE* ENDOTOXIN

2 *Serological Multispecificity and Other Properties of Phenol Water Preparations*

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Serological multispecificity of the polysaccharide part (*a* determinant) of gonococcal endotoxin was confirmed, using phenol water preparations from three strains of *Neisseria gonorrhoeae* and corresponding antisera Results permitted the establishment of antigenic factor formulas identical to those established on the basis of experiments with aqueous ether endotoxin The determinant factors (*a* factors) of the endotoxin are carried by the same molecular complex Haemagglutination inhibition studies with mono- and disaccharides in factor sera confirmed earlier results Galactose and *α* and *β* lactose blocked the combination of three of the *a* factors with corresponding antibodies

Endotoxin extracted with aqueous ether from three strains of *Neisseria gonorrhoeae* (strains 8551, V and VII) has one antigenic determinant of carbohydrate nature (determinant *a*) and another of protein nature (determinant *b*) (5, 7) The *a* determinant comprises several antigenic specificities (*a* factors) By indirect haemagglutination techniques using these three gonococcal strains and corresponding antisera, six *a* factors could be demonstrated (7)

While aqueous ether extracted endotoxin contained 82–88 per cent of protein and approximately 10 per cent of lipid and carbohydrate (6, 7), phenol water preparations from the same strains were composed mainly of carbohydrate and lipid (8)

The protein determinant *b* was present in phenol water preparations from strains V and VII but not from strain 8551 (8) All three preparations cross reacted with the *a* determinant of the corresponding aqueous ether extracted endotoxin and tended to be more active with respect to this determinant (8) The latter observation corresponds well with the higher content of carbohydrate in the phenol water preparations

Our findings indicate that phenol water preparations may be better suited for immunochemical analyses of the carbohydrate component of gonococcal endotoxin than aqueous ether preparations The serological cross-reactivity and the multispecificity of the *a* determinant of gonococcal endotoxin therefore have been reexamined using phenol-

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titioners who had provided a positive CA test were requested to forward clinical data of the patients The representation and randomization was inherent in the availability of such data from between 30 and 40 per cent of all CA positive patients found in each trimester

From the percentage of anti *M pneumoniae* positive sera in these materials the probable number of anti *M pneumoniae* positive out of all CA positive sera was calculated

RESULTS

The number of sera received annually for CA titration increased gradually from about 4000 in 1958 to about 7000 in 1969, with only minor fluctuations The number received each month showed regular seasonal variations it was high during winter, culminating at the turn of the year, and low in the summer months (Table 1 and Fig 1) It showed a quite good correlation with the number of notified cases of all types of pneumonia for the whole country[1]

However, the frequency of CA positive sera varied considerably both from year to year and from season to season (Table 1 and Fig 1) As a general rule, CA positive reactions were most common during winter

The probable number of anti *M pneumoniae* positive sera calculated for each trimester is shown in Table 1, while that for each month is plotted as a curve in Fig 1 This curve has four major peaks that indicate accumulation of anti *M pneumoniae* positive sera in the winters 1958/59, 1962/63 1963/64 and 1967/68 All four peaks fell within and more or less filled out, major peaks of the curve for CA positive sera During the intervals positive anti *M pneumoniae* sera were demonstrated most of the time However in some of the months mainly during spring and summer none of the CA positi e sera tested were found to be positive in the anti *M pneumoniae* tests In the months of [illegible] November and December the in[illegible]nce [illegible] generally slightly higher than in the other months of the year (Fig 1)

If the annual seasonal waves are overlaid the estimated frequency of positive anti *M pneumoniae* sera in the 48 trimesters (Table 1, last column) will be seen to correspond well with three waves at intervals of 4½ years By least square multiple regression analysis the following equation is obtained[2]

$$\log y = 1\ 25 + 0\ 66 \sin (t \times 20°) - 0\ 14 \cos (t \times 20°) + 0\ 12 \sin (t \times 90°) + 0\ 37 \cos (t \times 90°)$$

where log y is the logarithm of the estimated frequency in each trimester t, (t = 1, 48) This 'explains 62 per cent of the variation The first set of sin and cos presumes a periodicity of 18 trimesters (4½ years), the second set an annual wave (every fourth trimester)

The serological material collected from March 1968 to December 1969 represented 493 patients (Table 2) It was found that *A* A distribution according to diagnosis revealed 370 cases of pneumonia 49 cases of respiratory illness without pneumonia and no respiratory illness in 74 patients in whom there was a variety of diagnoses including haematological disorders *B* 419 or 85 per cent, of the patients had an acute respiratory illness *C* 118 (28 per cent) of these 419 patients had serological evidence of *M pneumoniae* infection They comprised 24 per cent of all patients in the material *D* All 118 patients with evidence of *M pneumoniae* infection had pneumonia *i e* the presence of antibodies to *M pneumoniae* was in no case associated with respiratory illness without pneumonia —It can be seen from Fig 1 that this material dated from a period in which there were only minor variations in the incidence of positive anti *M pneumoniae* test

During the 12 year period, 9 major epi

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[1] The material of [illegible] cases of pneumonia not reported here was [illegible]dly provided by the Danish National Health S[illegible]vic Department of Medical Statistics

[2] [illegible]ggested by Dr Johs Ipsen Henry Phipps Insti[illegible]te, Phil[illegible]lphia U S A

TABLE 1 *Calculations of the Number of Anti M Pneumoniae Positive Sera in a 12 Year Material Presented in 3 Month Periods*

Year	Trimester	Number of sera tested for cold agglutinins (CA)		CA pos sera tested for anti M pneum		Calculated anti M pneum pos out of CA pos
		Total	Positive	Total	Positive	
1958	I	1205	144	45	11	35
	II	889	78	42	12	22
	III	819	110	37	16	48
	IV	1482	490	75	50	327
1959	I	1539	342	62	25	138
	II	1121	82	32	8	21
	III	635	30	12	3	8
	IV	924	70	25	4	11
1960	I	1246	52	21	1	3
	II	935	43	27	2	3
	III	683	24	23	3	4
	IV	907	78	21	4	15
1961	I	946	75	31	3	7
	II	976	74	32	0	<2
	III	755	86	33	1	3
	IV	1177	203	40	1	5
1962	I	1419	158	70	5	11
	II	1029	79	53	10	15
	III	813	117	62	5	9
	IV	1336	290	65	42	187
1963	I	1674	293	64	23	105
	II	1244	125	60	26	54
	III	1142	158	91	46	80
	IV	1867	447	60	39	291
1964	I	2296	747	60	19	237
	II	1598	141	62	7	16
	III	1092	72	58	15	19
	IV	1261	196	60	18	59
1965	I	1599	194	84	10	23
	II	1414	297	70	3	13
	III	1216	283	60	3	14
	IV	1703	336	74	6	27
1966	I	1831	316	58	0	<5
	II	1690	233	52	0	<5
	III	1164	148	69	1	2
	IV	1702	280	50	4	22
1967	I	1726	251	56	5	22
	II	1434	147	84	7	12
	III	1191	176	86	19	39
	IV	2353	552	114	53	257
1968	I	2605	441	131	40	135
	II	1642	237	86	22	61
	III	1349	146	70	17	36
	IV	2223	313	142	41	90
1969	I	2316	20[illegible]	153	27	37
	II	1776	15[illegible]	112	8	11
	III	[illegible]18	[illegible]6	48	7	10
	IV	[illegible]325	171	118	23	34

tions This is reflected in the concurrence of these two reactions as an indication of actual or recent illness (23)

Furthermore, if one reviews the results of previous studies from March 1966 to March 1968 (23) mentioned in the introduction, the incidence of *M pneumoniae* infection found by examination of patients with both positive and negative CA tests strongly supports the present calculated figures for that period (Fig 1) The low calculated number agrees with the low incidence of *M pneumoniae* infection among hospitalized patients during the first year (23)

The calculated figures for positive anti-*M pneumoniae* sera do not permit of the determination of the exact number of *M pneumoniae* infections at any time of the period This is mainly bound up with the lack of information as to how many of the notified cases of pneumonias were p a p , and how many of the patients suffering from these pneumonias were tested for CA

However, the results of this study form a reasonable basis for the estimation of the chronological variations in the incidence of *M pneumoniae* infection It is conceivable that the three waves of the calculated frequency of positive sera closely mirror three major outbreaks or epidemics of the disease in this country Both intervals cover 4½ years The middle epidemic from autumn 1962 to winter 1964 shows a temporary depression during the spring and summer of 1963, this is due to the constantly reappearing low incidence in these seasons

The serological material collected from March 1968 to December 1969 represented 493 patients from a period in which there were only minor variations in the incidence (Table 2 and Fig 1) The distribution of these patients according to diagnosis may reflect the situat the main part of the 12 year mater the major peaks of epidemics

It is remarkable the concurrence of a positive anti-*M pneu ia* test and a positive CA test was found e lusively in patients with pneumonia 49 (13 per cent) out of 375 patients with a negative anti-*M pneumoniae* test and a positive CA test suffered from acute respiratory illness without pneumonia (Table 2) Since *M pneumoniae* infection may lead to both upper and lower respiratory illness without pneumonia (15), these data would seem to indicate that such infections do not give rise to significant CA production Several studies have shown that the development of CA is directly correlated to the severity of illness and the involvement of the lungs (4, 7, 25, 1), but lack of this correlation has also been reported (8, 13) The *M pneumoniae* organisms must presumably exert their pathogenic activities in the lung before the production of CA is triggered off during the infection concerned

An interesting point is the high number of CA positive sera occurring in certain periods without indication of either *M pneumoniae* or influenza epidemics The accumulation of such sera as peaks on the curve seen in Fig 1 is remarkable It is most probable that the illness underlying these peaks was for each peak dominated by a single infectious agent which in the majority of patients gave rise to CA production This or these pathogens are so far unknown

The data presented here concerning conditions in Denmark are discussed in relation

TABLE 2 Mycoplasma pneumoniae *Antibodies Related to Clinical Diagnosis in Patients with Positive Cold Agglutinin Tests (March 1968-December 1969)*

Clinical diagnosis	*Number of patients* Anti *M pneumoniae* FAT positive	*Number of patients* Anti *M pneumoniae* FAT negative	Total
Pneumonia	118	252	370
Acute resp illness without pneumonia	0	49	49
No resp illness	0	74	74
Total	118	375	493

to data obtained from Stockholm Oslo and Helsinki *Biberfeld et al* (2) published a study on *M pneumoniae* infection in the Stockholm area during the period February 1964 to March 1966 There were no significant fluctuations in the incidence By extending the study to June 1968 they found a significantly higher frequency of cases in October–November 1967 (3) In Oslo, *Eng* (9) investigated sera from 151 patients hospitalized with pneumonia in the period June 1964 to June 1966 *M pneumoniae* infection was diagnosed in only 10 patients The distribution of these cases showed no significant accumulation within the period (10) From Helsinki *Jansson* reported the results of her serological investigations of the infections from September 1962 to March 1970 (Table 3) An increased number of cases was recorded both during the winter 1962/63 and in the years 1967 and 1968 (18)

TABLE 3 *Occurrence of* Mycoplasma pneumoniae *Infections in Helsinki from 1962 to 1970**

Period	Number of patients	
	Investigated	*M pneum* infection
September 1962		
April 1963§	246	40
May–December 1963	14	0
1964	35	1
1965	55	0
1966	146	0
1967	490	47
1968	484	55
1969	538	3
January March 1970	15	0

* The data were kindly provided by Dr *Elli Jansson*

§ Published in Brit med J *I* 142–145 1964 by *Jansson E et al*

In his studies on the *M pneumoniae* infection among children in Copenhagen *Hornsleth* (17) found a high incidence of the illness in the fourth *trimester of 1963*

The results from Sweden, Norway, Finland and Denmark may indicate a common epidemiology of *M pneumoniae* disease in Scandinavia

In the papers published by *Grayston et al* (summarized in 15) on the incidence of *M pneumoniae* infection in Seattle, U S A, a major epidemic was registered in the winter 1966/67, minor outbreaks occurred from January to July 1966 In the remainder of the period 1964–1967 the rates were endemic with only small variations They also noted a low incidence of *M pneumoniae* infection during an influenza epidemic (14) *Evans* and co workers (11) presented serological investigations of *M pneumoniae* epidemiology among students admitted to an infirmary in Wisconsin during a 12 year period from 1953 to 1965 A cyclic pattern was seen with an increase of both total pneumonia and *M pneumoniae* pneumonia every 4 to 5 years Monthly analysis revealed October, November and January peaks These results are consistent with those of the present study

Hers & *Masurel* (16), who studied the *M pneumoniae* infection among civilians in the Netherlands, found that it occurred throughout the year Although their material, as they acknowledge was somewhat biased by the selection of patients there was evidence suggestive of a wavelike incidence during the years studied

In Switzerland *Krech* & *Paccaud* (19) compared the incidence of infections due to *M pneumoniae* in two geographic areas In the central and eastern (German speaking) parts of the country the incidence was high in 1965 while this was the case in 1966 in the western (French speaking) parts The authors do not offer any explanation of this phenomenon which probably reflects poor contact between the two populations

In military camps, the incidence of *M pneumoniae* illness has also been found to fluctuate (5, 6, 12)

Before the international aspects of the epidemiology and ecology of *M pneumoniae* infection can be estimated, long term studies of large populations in adjacent geographic areas will be necessary

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STUDIES ON ENDO-β-N-ACETYLGLUCOSAMINIDASE, STAPHYLOLYTIC PEPTIDASE, AND N-ACETYLMURAMYL-L-ALANINE AMIDASE IN LYSOSTAPHIN AND FROM *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus, strain M18, produces extracellular bacterial cell wall degrading enzymes of three different classes endo-β-N acetylglucosaminidase, endopeptidase, and N-acetylmuramyl L-alanine amidase All are basic proteins, adsorbed on CM Sephadex with isoelectric points (pI) in the range pH 9 5 – 10 5 Separation of lysostaphin by isoelectric focusing in a very shallow pH gradient resolved these enzymes that have not previously been separated The charge of each of these enzymes is similar to that on the corresponding ones from strain M18 The isoelectric point of the staphylolytic peptidase was 10 4 the pI of the amidase 9 8, and the pI of the glucosaminidase 9 5 Separation by Sephadex G 100 gel chromatography also showed the similarity in size of the corresponding enzymes from the two sources Digestion of staphylococcal peptidoglycan with the purified staphylolytic peptidase with a pI of 10 4 and the staphylococcal endo β N acetylglucosaminidase of strain M18 gave a high recovery of disaccharide peptide monomer which was purified by Sephadex G 50 and G 25 chromatography and used as substrate for the amidases Neither the amidase of *S aureus* nor the one of lysostaphin was found to be bacteriolytic *per se* The heterogeneity of the bacteriolytic activity of strain M18 was further studied by isoelectric focusing and acrylamide electrophoresis Component A (pI 9 5) and B (pI 7) are probably isoenzymes of the endo β-N acetylglucosaminidase, since they had similar properties and were not separable by molecular sieve chromatography Immunodiffusion experiments show that the glucosaminidase of *S aureus*, strain M18, and the corresponding enzyme of lysostaphin are probably not structurally related which is remarkable due to the rare occurrence of bacteriolytic endo-β N acetylglucosaminidases in nature

Degradation of the basic structure of all bacterial cell walls [illegible] he mucopeptide, peptidoglycan, glycop[illegible] r murein can be brought about b[illegible] three [illegible] lasses of enzymes (*1*) glycosidases or [illegible] minidases which [illegible] the glycan polym[illegible] acetylmuramic acid and N-acetylglucosamine, (*2*) amidases which split the bond between the D lactyl moiety of N-acetylmuramic acid and the N terminal amino acid (usually L-alanine) of the peptide attached to it, and (*3*) endopeptidases which split bonds within these peptides and their cross bridges (18, 19 49)

Recently *Wadström & Hisatsune* (59, 60)

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were able to purify and characterize a bacteriolytic endo β N acetylglucosaminidase from *S aureus*, strain M18 A semi purified enzyme was shown to contain a peptidase and possibly an N acetylmuramyl L-alanine amidase activity (60) It was proposed that these enzymes might be the same or similar to the glucosaminidase, peptidase, and amidase in lysostaphin (6, 45) which is produced by a strain of *S epidermidis*, strain K 6-WI (45)

These enzymes have not previously been completely separated by conventional techniques Preliminary experiments with isoelectric focusing revealed very similar isoelectric points of the lysostaphin enzymes and the endopeptidase and amidase of strain M18 By using specially synthesized carrier ampholytes (aliphatic polyamino polycarboxylic acids) with isoelectric points in the pH range 9 5 – 11, it was possible to extend the pH gradients to permit separation of these enzymes

The staphylolytic endopeptidase with a high specificity of action against the pentaglycine bridge of the *S aureus* cell walls was separated from the amidase and glucosaminidase present in the preparation of lysostaphin This peptidase was used together with the endo β N acetylglucosaminidase from strain M18 for digestion of staphylococcal peptidoglycan

Purification of the cell wall digest of *S aureus*, strain 3528, by Sephadex chromatography yielded a disaccharide peptide monomer which was used as a substrate for studies on the endopeptidase free N acetylmuramyl L alanine amidases This is probably the first time that cell wall degrading enzymes of all three classes, hexosaminidases peptidases and amidases have been separated and characterized in one strain of *S aureus* The bacteriolytic activity in culture supernatants of strain M18 was assayed on whole cells of *M lysodeikticus* It was possible to resolve multiple molecular forms by isoelectric focusing The heterogeneity was further studied in this investigation in order to show whether these activities are caused by different enzymes or are artifacts of the isolation procedures

MATERIALS AND METHODS

Materials Lysostaphin (5619 7 A and M 2 26) and purified hexosaminidase (178-74) were kindly donated by Dr *P A Tavormina* and co-workers at Mead Johnson Evansville, Ind, USA Acrylamide was purchased from Eastman Kodak Chem, Rochester, N Y, USA Carrier ampholytes Ampholine® pH 3 – 10 and pH 8 – 10, were used (LKB Produkter AB, S 161 25 Bromma, Sweden) Glycerol sucrose, and silica gel (kiselguhr G) were obtained from Merck, Darmstadt, Germany Sephadex and dextran blue 2000 were purchased from [illegible] lyo [illegible] white [illegible] ne c, [illegible] itro fluorobenzene (FDNB) and DNP amino acids were purchased from Sigma Chem Comp, St Louis, Mo, USA Sperm whale myoglobin was purchased from Mann Res Labs, New York N Y USA and human haemoglobin (2x crystallized) from Nutritional Biochem Corp, Cleveland Ohio, USA Human serum albumin and human gammaglobulin were gifts from KABI, Stockholm Sweden Polyethyleneglycol 20M (PEG) technical grade was purchased from KEBO Stockholm, Sweden

Bacterial strains S aureus, strain M18, was used for production of the bacteriolytic enzymes (4, 59) Whole cells and cell walls of strain 3528 of *S aureus* were used as substrates for a given enzyme sample

Production and purification of the S aureus *enzymes* The bacteriolytic enzymes of *S aureus*, strain M18 were produced in CCY1 medium (3,4) in continuous culture under automatic control of pH temperature aeration agitation and foam (24) The outflow was collected, rapidly cooled, and centrifuged (8000 x g 10 min) every third hour and the supernatant was dialyzed by a continuous procedure against 0 03M ammonium acetate (63)

(A) Twenty litres of culture supernatant were appl ed on a column packed with CM Sephadex C 25 and then eluted with 1 500 ml of 0 2M phos[illegible]te buffer pH 7 5 containing 0 3M NaCl This [illegible] was dialyzed as before and lyophilized A po[illegible]on of 20 mg was dissolved in 30 ml of glycine 1 per cent w/v) and subjected to isoelectric focu[illegible]ing

(B) Dialyzed culture supernatant (80 ml) was freeze dried and dissolved in 2 ml distilled water This material was chromatographed on a Sephadex G-50 column [illegible] × 120 cm) equilibrated with one of the follo[illegible]ng buffers (a) 0 1M sodium acetate buffer pH 6 5, (b) the same buffer containing 0 5M sod[illegible]m chloride Crude supernatant [illegible] also conce[illegible]trated ten times by poly[illegible]hylen[illegible] (PEG, see below) and chrom[illegible]phed [illegible] Sephadex G 100 column (2 × [illegible]n) [illegible]

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STUDIES ON ENDO-β-N-ACETYLGLUCOSAMINIDASE, STAPHYLOLYTIC PEPTIDASE, AND N-ACETYLMURAMYL-L-ALANINE AMIDASE IN LYSOSTAPHIN AND FROM *STAPHYLOCOCCUS AUREUS*

T Wadström and O Vesterberg

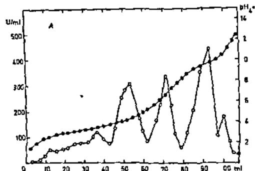
Department of Bacteriology, Karolinska institutet S 104 01 Stockholm 60 Sweden

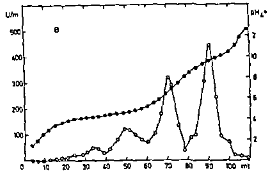
Staphylococcus aureus strain M18 produces extracellular bacterial cell wall degrading enzymes of three different classes endo β N acetylglucosaminidase, endopeptidase and N acetylmuramyl L alanine amidase All are basic proteins adsorbed on CM Sephadex with isoelectric points (pI) in the range pH 9 5 10 5 Separation of lysostaphin by isoelectric focusing in a very shallow pH gradient resolved these enzymes that have not previously been separated The charge of each of these enzymes is similar to that on the corresponding ones from strain M18 The isoelectric point of the staphylolytic peptidase was 10 4 the pI of the amidase 9 8, and the pI of the glucosaminidase 9 5 Separation by Sephadex G 100 gel chromatography also showed the similarity in size of the corresponding enzymes from the two sources Digestion of staphylococcal peptidoglycan with the purified staphylolytic peptidase with a pI of 10 4 and the staphylococcal endo β N acetylglucosaminidase of strain M18 gave a high recovery of disaccharide peptide monomer which was purified by Sephadex G 50 and G 25 chromatography and used as substrate for the amidases Neither the amidase of *S aureus* nor the one of lysostaphin was found to be bacteriolytic *per se* The heterogeneity of the bacteriolytic activity of strain M18 was further studied by isoelectric focusing and acrylamide electrophoresis Component A (pI 9 5) and B (pI 7) are probably isoenzymes of the endo β N acetylglucosaminidase since they had similar properties and were not separable by molecular sieve chromatography Immunodiffusion experiments show that the glucosaminidase of *S aureus*, strain M18, and the corresponding enzyme of lysostaphin are probably not structurally related which is remarkable due to the rare occurrence of bacteriolytic endo β N acetylglucosaminidases in nature

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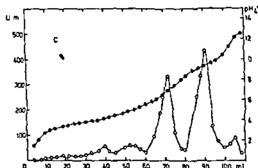


Fig 6 (A) Separation of crude culture supernatant of strain M18 by isoelectric focusing in a glycerol gradient containing Ampholine pH 3-10 Three ml fractions were collected, pH of each fraction was measured (●—●), and bacteriolytic activity against *M lysodeikticus* whole cells (○—○) was assayed for Separation of the corresponding material (B) after dialysis against 0 1 M phosphate buffer, pH 7 0, containing 0 5 M NaCl, (C) after chromatography on a Sephadex G 150 column

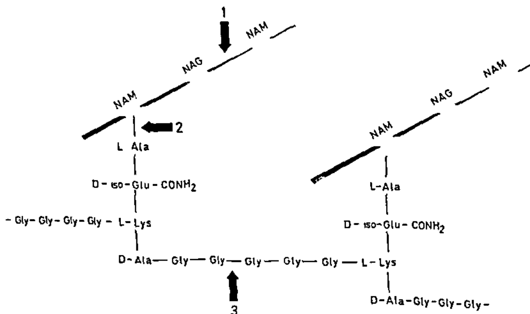


Fig 7 A fragment of the peptidoglycan of *S aureus* showing points of cleavage by (1) endo-β N acetyl glucosaminidase, (2) N acetylmuramyl L alanine amidase, (3 endo-peptidase of *S epidermidis*, strain K-6 1, and *S aureus*, strain M18 Abbreviations NAG, N acetylglucosamine NAM, N acetylmuramic acid

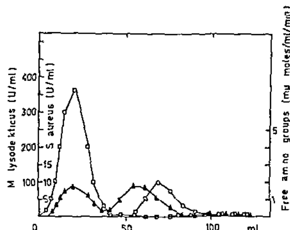


Fig 4 Separation of crude culture supernatant on a column packed with Sephadex G 50 fine (2 × 120 cm) Elution was carried out with a 0 1 M sodium acetate buffer, pH 6 5, containing 0 1 mM dithioerythritol and 0 5 M NaCl at a flow rate of 20 ml/h The void volume was 180 ml and dextran blue was eluted in the first 30 ml Bacteriolytic activity was assayed on whole cells of *M lysodeikticus* (□——□) and *S aureus* (▲——▲) The N acetyl muramyl L alanine amidase activity was determined as the amount of released free amino groups from disaccharide peptide monomer (○——○)

bacteriolytic activity concentrated from culture supernatant of strain M18 by rotary evaporation is shown in Fig 4 A plot of the mol wts of some standard proteins and of the enzymes studied is shown in Fig 5

Immunodiffusion Endo β N-acetylglucosaminidase from lysostaphin (178–74) was further purified by isoelectric focusing (Fig 2B) Two polyvalent staphylococcal antisera "011" and anti M18" (61) did not give precipitate with the purified enzyme diluted in the range 0 5 – 0 01 mg/ml, while the glucosaminidase of strain M18 purified according to Table I in reference 59 gave a strong line in this range of dilution against the undiluted '011' and M18 antiset Thus, the glucosaminidases of strain M18 and of lysostaphin seem to be antigenically nonrelated

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After chromatography on Sephadex G 150 in 0 1M phosphate buffer, pH 7 0, containing 0 5M NaCl, the component C disappeared almost entirely (Fig 6C), while the components A and B remained

Properties of the components A, B, and C Both components A and B released reducing sugar from *M lysodeikticus* cell walls and

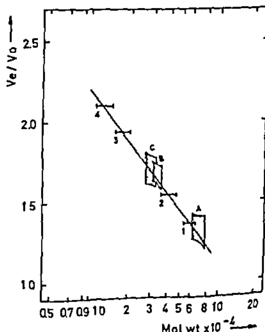


Fig 5 Estimation of the molecular weights of endo-β N acetylglucosaminidase (A), staphylolytic peptidase (F), and N acetylmuramyl L-alanine amidase from *S aureus*, strain M18 by chromatography on Sephadex G 100 Reference proteins (1) human serum albumin (2) ovalbumin, (3) myoglobin, chrome c

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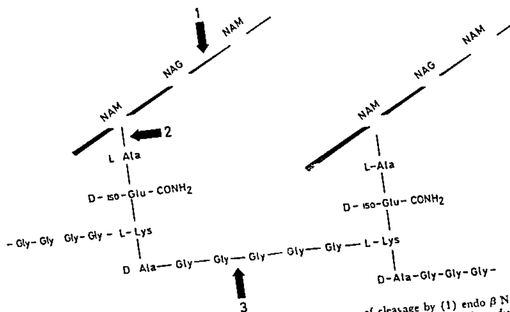


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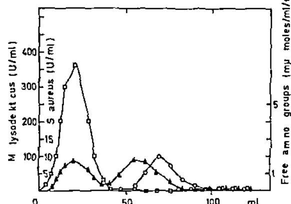


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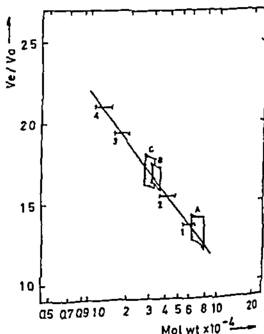


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Acta path microbiol scand Section B 79, 265-274, 1971

ELIMINATION OF GENETIC ELEMENTS GOVERNING COMPETENCE IN TRANSFORMATION OF *NEISSERIA MENINGITIDIS* BY TREATMENT WITH ETHIDIUM BROMIDE AND ACRIFLAVIN

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Competence in the DNA mediated transformation of *Neisseria meningitidis* Strain M1 and Strain Ne15 depends upon a heritable factor called *cp* Ethidium bromide was found to be a powerful agent in eliminating competence in genetically competent (cp^+) variants of the two strains The strain Ne15 could also be rendered genetically incompetent (*cp*) by acriflavin at low frequencies When suspensions of meningococci were treated with ethidium bromide, the frequency of streptomycin resistant mutants could be increased around ten fold above the spontaneous frequency With acriflavin the frequency could be increased approximately two fold Both drugs seemed preferentially to mutagenize at the replication point of the chromosome The mutagenic effect as well as the killing effect of ethidium bromide and acriflavin was higher in cp^+ variants than in *cp* ones. In growing cultures a mutagenic effect of ethidium bromide could also be demonstrated, whereas no significant mutagenic effect of acriflavin could be found The inhibiting effect of the two drugs seemed to be the same in growing cultures of cp^+ variants as it was in cultures of *cp* variants The findings from *N meningitidis* have been discussed in relation to the action of these dyes on various genetic determinants of plasmid type in other microbes

...rdine dyes such as proflavin and acri... are mutagenic for bacteriophages, but ... been reported to be non mutagenic for bacteria (5) Many genetic determinants of antibiotic resistance in *Enterobacteriaceae* and in *Staphylococcus* which have been shown to belong to extrachromosomal elements can be eliminated by acridi...es, though generally at low frequen...

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flavin is also an effective agent for eliminating the F-factor in *Escherichia coli* K12 (1, 6) Acridine molecules seem to be inserted or intercalated between adjacent base pairs of DNA causing an extension and unwinding of the phosphodiester backbone (16)

Ethidium bromide is a trypanocidal dye which has also been found to ... properties (14) *Bouanchau*... showed that ethidium bromi... ful agent in eliminating

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Acta path microbiol scand Section B 79, 275-280, 1971

DELAYED HYPERSENSITIVITY IN THE GUINEA PIG IMMUNIZED WITH KILLED TUBERCLE BACILLI IN ADJUVANT

I *Development of Peritoneal Cell Migration Inhibition, Skin Reactions and Antibodies to Tuberculin Purified Protein Derivative*

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Guinea pigs were immunized with 2 4 mg of killed tubercle bacilli in adjuvant From 4 days to one year after sensitization Tuberculin Purified Protein Derivative (PPD) was used to test migration inhibition (25 γ/ml), skin reactivity (10 γ) and antibodies Delayed 24 hours skin reactions became positive slightly earlier than migration inhibition hemagglutinating antibody and 4 hour skin reactivity Migration inhibition was very strong 4 and 6 months and one year after sensitization, when the animals also had strong 4 and 24 hour skin reactions and hemolysing antibody

Rich & *Lewis* were the first to describe that cells from spleens and lymph nodes of tuberculin sensitive animals showed poor migration in the presence of tuberculin (17) Later *George* & *Vaughan* studied the migration of guinea pig peritoneal exudate cells out of capillary tubes and showed that the inhibition of migration by tuberculin or egg albumin paralleled delayed skin reactivity to these antigens (14)

Using this capillary tube technique, *David et al* showed that as few as 2 5 per cent of peritoneal cells from sensitive guinea pigs mixed with normal cells, caused inhibition of the whole population and that the sensitive cells must be viable (8) Inhibition does not occur if the protein synthesis of the cells is blocked by puromycin (10) Peritoneal exudate consists mainly of macrophages with a minority of lymphocytes and polymorphonuclear leucocytes *Bloom* & *Bennett* removed the lymphocytes by tissue culture methods and showed that the remaining cells were no longer inhibited by antigen, but that the readdition of even a small amount (0 6 per cent) of peritoneal lymphocytes from sensitive animals resulted in migration inhibition (3) It was found that sensitive lymphocytes interacting with specific antigen produce a soluble, nondialyzable material that mediates the inhibition of macrophage migration (3, 11)

A comparison between the development of migration inhibition, skin reactions and serum antibody has only been made during the first 3 weeks after immunization (16) In the present work this was carried out up to one year after sensitization

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a + + + migration (no inhibition)



b + + migration (slight inhibition)



c + migration (clear inhibition)



d 0 migration (total inhibition)

Fig 1 Photographs of migration chambers demonstrating the expression of the extent of migration Magnification × 10

MATERIALS AND METHODS

Animals Male albino guinea pigs weighing 350 g to 500 g were used

Sensitization Mycobacterium tuberculosis C DT, PN strains (heat killed, washed, dried) were mixed in a concentration of 4 mg/ml in adjuvant, which consisted of 90 per cent Bayol F (Esso) and 10 per cent Arlace A The animals were sensitized by an injection of 0 1 ml into each footpad, and 0 2 ml intracutaneously into the back of the neck

Migration tests Tests were carried out using the technique described by *David* (7), slightly modified A peritoneal exudate was induced by an intraperitoneal injection of 30 ml of 2 5 per cent starch gel in physiological saline (Starch- Hydrolyzed, Connaught Laboratories, Toronto) Three days later the exudate cells were collected and washed twice in Hanks salt solution They were suspended in Eagle s medium containing 15 per cent normal inactivated guinea pig serum and taken

portions containing the cells were placed in tissue culture chambers two capillaries per chamber The chambers were filled with Eagle s medium containing 15 per cent normal inactivated guinea pig

serum with 25γ PPD/ml (Tuberculin Purified Protein Derivative, Parke Davis et Company, Detroit) or without antigen at least two chambers of each They were examined microscopically after 20 to 24 hours incubation at 37°C Migration areas were photographed using a 2 5 × objective The photographs were used for comparing the migration areas and expressing the extent of migration as follows The cells in the control chambers without antigen migrated out of the capillary tube in a lacy fan like pattern (Fig 1 a), this was expressed as + + + + migration The cells slightly inhibited by antigen appeared in a smaller area with a smooth clearly defined border (Fig 1 b), this was expressed as + + migration When the inhibition was clear the cells appeared as a dense clump (Fig 1 c), this was expressed as + migration When the inhibition was total the extent of migration was 0 (Fig 1 d)

Skin testing The animals were tested intradermally with 10 γ PPD in 0 1 ml of physiological saline The tests were made 24 hours before the peritoneal cells were collected for migration tests and the reactions were measured in some animals at 4 hours and in all animals at 24 hours just before killing The diameter of erythema was measured in millimeters and the intensity of induration was expressed between 0 and + + + + after palpation

In order to study the histology of 24 hour skin tests biopsies were taken from some skin test sites and fixed in 10 per cent neutral formalin They were dehydrated embedded in paraffin and 4 μ sections were cut and stained with Giemsa solution at pH 4 5

Serum antibody determination Blood samples were taken at the same time as peritoneal exudates were collected. Antibody to PPD was titrated by passive hemagglutination as described by *Boyden* (6) The amount of PPD used to sensitize tanned sheep red blood cells was 2 5 mg/1cc packed cells After hemagglutination titers had been read passive immune hemolysis titers were determined by adding guinea pig complement and incubating at 37°C as described by *Bloch et al* (2) The highest dilution giving visible hemolysis was taken as the end point

Sensitivity to mercaptoethanol treatment was determined by adding 2 mercaptoethanol (Fluka AG Buchs SG Switzerland) to each tube of serum dilution giving a final concentration of 0 05 M and incubating in a water bath at 37°C for 30 min before adding the sensitized cells (18)

RESULTS

Migration tests (Fig 2) The migration of peritoneal cells of normal guinea pigs was unaffected by 25 γ PPD/ml [illegible] animals tested, the remaining 5 [illegible] migration in the presence of [illegible]

Migration tested at 4 days after [illegible]tion was unaffected by PPD [illegible] animals tested, in one [illegible] (+ + + +) and in one [illegible] (+ + migration) In the [illegible] no inhibition was seen at 5 and [illegible]

At 7 days migration [illegible] between + and + + + In [illegible] of the animals migration was [illegible]ited (+ migration) At 14 days [illegible] was similar In 4 out of [illegible] migration was not inhibited [illegible] it was totally inhibited. At [illegible] still some animals show bition

the animals produced only serum antibody, and that the migration of normal cells after incubation in sera containing serum antibody or in sera from animals exhibiting delayed hypersensitivity, was not inhibited by antigen However, *Amos et al* (1) and *Hesse et al* (15) have produced migration inhibition by incubating normal macrophages in immune sera which contained antibody cytophilic for macrophages using PPD as antigen

According to the present results it is possible that from 28 days up to one year after sensitization, serum antibody magnifies 24 hour skin reactions because of the simultaneous expression of 4 hour skin reactivity Whether cytophilic antibody present on the surface of macrophages contributes to the strong migration inhibition reactions remains to be seen

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This study was supported by grants from the *Sigrid Jusélius Foundation* and from the *Finnish National Research Council for Medical Sciences* to Dr *T U Kosunen* I am grateful for the excellent technical assistance of Mrs *Maire Laakso* Miss *Marja Hirvonen* and Miss *Irmeli Rantala*

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Acta path microbiol scand Section B 79, 281 284, 1971

DELAYED HYPERSENSITIVITY IN THE GUINEA PIG IMMUNIZED WITH KILLED TUBERCLE BACILLI IN ADJUVANT

2 The effect of Tuberculin Purified Protein Derivative Concentration on the Inhibition of Peritoneal cell Migration

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Guinea pigs were immunized with 2 4 mg of killed tubercle bacilli in adjuvant The effect of Tuberculin Purified Protein Derivative (PPD) concentration on peritoneal cell migration was studied at various times from 7 days to 6 months after sensitization At 7 days migration was inhibited significantly in the presence of 25 γ PPD/ml but not in the presence of 0 8 γ PPD/ml At 28 days migration was inhibited highly significantly also with the lower dose Later after sensitization inhibition was even stronger, at 6 months 0 01 γPPD/ml was also inhibitory

Using bovine gammaglobulin as antigen *David* (1) has shown that the inhibition of peritoneal cell migration in the guinea pig is dependent on antigen concentration

In my previous work (3) the development of peritoneal cell migration inhibition in the guinea pig was followed after sensitization with killed tubercle bacilli in adjuvant using only one antigen concentration (25 γ PPD/ml) This caused some degree of migration inhibition in many animals by 7 days after sensitization At 4 and 6 months inhibition was almost total In the present investigation the effect of antigen concentration on migration inhibition at various times after sensitization is studied

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MATERIALS AND METHODS

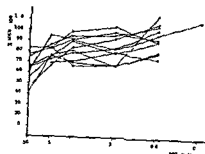
Animals Male albino guinea pigs weighing 350 g *to 500 g were used Some of the animals were the* same as those of the previous work (3)

Sensitisation, skin testing and antibody titration were carried out as described previously (3)

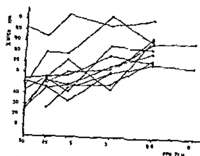
Migration inhibition technique was described *in* the previous article (3), except that the area *of* cell migration was now measured by planimetry The images of migrated cells in the chambers were first projected (magnification × 30), and then traced on transparent paper The extent of migration was expressed as per cent migration with antigen =

$$100 \times \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}}$$

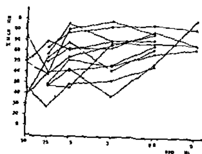
The average area of migration was calculated with at least 4 capillary tubes except at a concentration of 50 γ PPD/ml, when only two capillaries were made The PPD amounts γ/ml were 50, 25, 12 5 3 1, 0 8 and in some tests 0 2, 0 05 and 0 01



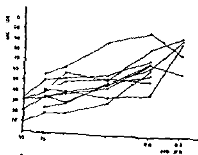
a normal animals



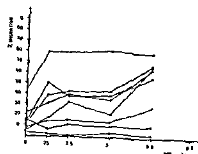
b 7 days after sensitization



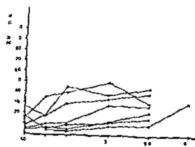
c 14 days after sensitization



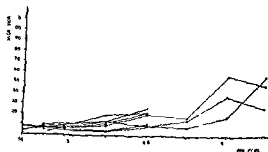
d 28 days after sensitization



e 2 months after sensitization



f 4 months after sensitization



g 6 months after sensitization

Fig 1 The effect of various amounts of PPD on the migration of peritoneal cells of guinea pigs at various times after sensitization with killed tub rcle bacilli in adjuvant Each line represents one animal

RESULTS

Normal animals Fig 1 a and Fig 2) The h est antigen concentratio used was 50 γ P ml It was four inhibitory in m normal animals average per cent mi s (6 st n d error) Small er conce tr tions of PPD also had some in hibitory t aver ge per cent migration using 2)/ml 78± using 12 5 γ PPD/m 3 3 a ng 0 8 γ PPD/ml 94 ±

7 ys af on (Figs 1

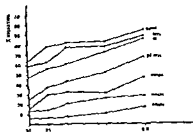


Fig 2 The effect of various amounts of PPD on the migration of peritoneal cells of guinea pigs at various times after sensitization with killed tubercle bacilli in adjuvant Each line is the average of the experiments

b and c and Fig 2) Using 25 γ PPD/ml individual animals had at 7 days very varied per cent migrations (between 27 per cent and 94 per cent average 56±5 8) When 12 5 γ PPD/ml was used average per cent migration was 60 ± 7 1 Even 0 8 γ PPD/ml was slightly inhibitory in some animals (variation between 69 per cent and 111 per cent average 85 ± 4 0) The average per cent migration differed significantly from that of normal animals (t test, P<0 01) in the presence of 25 γ PPD/ml and 12 5 γ PPD/ml but not in the presence of 0 8 γ PPD/ml

At 14 days all antigen concentrations used gave average per cent migration values which were somewhat higher than in animals tested at 7 days but as a whole the situation was nearly the same The average per cent migration with 25 γ PPD/ml was 63 ± 5 1 which differed significantly from that of normal animals but with 12 5 γ PPD/ml it was 77 ± 5 7 which was not significant Using 0 8 γ PPD/ml the average per cent migration was 86 ± 3 8

50 γ PPD/ml gave average per cent migration values of 48 ± 8 7 and 59 ± 9 0 at 7 and 14 days respectively these were not significantly different from those of controls

28 days (Fig 1 d and Fig 2) Average per cent migration using 25 γ PPD/ml was 38 ± 3 4 and with 0 γ PPD 7 ± 5 2 Both these s di d hig ificantly from those of no imals 0 00)

2 months and 2 his time aver igra 25 γ

PPD/ml and 0 8 γ PPD/ml were 30 ± 9 0 and 47 ± 10 3 respectively, and they differed highly significantly from those of normal animals Among the 8 animals tested there were one which differed from all others because per cent migrations with all antigen concentrations were within the range of normal animals 24 hour skin reaction of this animal measured 18 mm in diameter and + *induration and serum agglutinin titer* was 1 40 which were somewhat smaller values than usual at this time after sensitization

4 months (Fig 1 f and Fig 2) Average per cent migrations with 25 γ PPD/ml and 0 8 γ PPD/ml were 15 ± 4 2 and 28 ± 4 8 respectively

6 months (Fig 1 g and Fig 2) The inhibition was stronger than earlier average per cent migrations with 25 γ PPD/ml and 0 8 γ PPD/ml were 7 ± 0 9 and 17 ± 2 7 respectively Three animals were tested using smaller antigen concentrations 0 01 γ PPD/ml gave an average per cent migration of 44 ± 9 1

DISCUSSION AND CONCLUSIONS

The present results demonstrate that all antigen concentrations have stronger inhibitory effects at later times after sensitization than at earlier The sensitivity of the test thus increases during the course of sensitization At 7 days 25 γ PPD/ml is needed for inhibition but at 6 months even 0 01 γ PPD/ml is inhibitory This might follow from increased production of migration inhibitory factor due to increasing affinity for antigen of the receptor antibody on the surface of lymphocytes It might also be due to activation of lymphocytes by *antigen-antibody* complexes In addition *cytophilic antibody* on the surface of macrophages may contribute to migration *inhibition*

Peritoneal cells of many normal animals are nonspecifically inhibited by 50 γ PPD, which makes it impossible to use gh concentrations for testing animals at er times after sensitization Th re ult

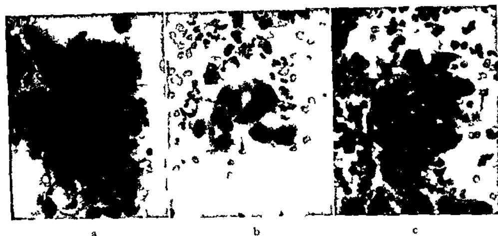
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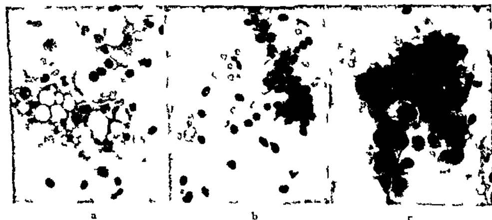
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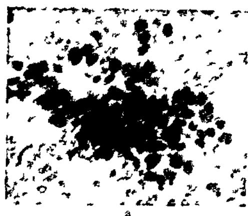
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F   g   n   t   on cell clus ers seen on sl de) but subse and c) Mastit s Clusters of h st ocytes obably der ed from ductal ep thel um



Fg 3 F l rocys c d ea e a) Gro p of three sud oval naked nucle b Plug of ductal ep hel ur ca c noma l ke cell among ben gn ep hel um a on a (H s olog c d agno s f brocys c d se



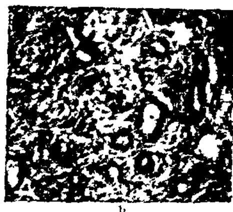


Fig 4 Fibrosing adenosis a) Slightly cellular atypia Malignancy s pected cyto ogically MGG ×400 b) Tissue section Van Gieson ×250





Fig 5 Fibroadenoma Solid plugs indicate strong intercellular cohesion some free oval naked nuclei are present a) MGG ×400 b) Papanicolaou stain ×400

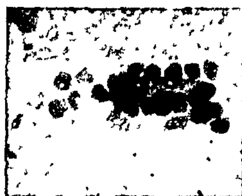
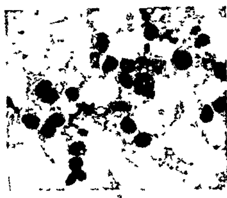




Fig 6 Smears fr Cell clusters cytolog excisional biopsy we carcinoma subsequent cases in which the cytologic report stated suspected malignancy egarded as indicative of carcinoma (The histologic diagnoses after ocystic disease in (a) and fibroadenoma in (b) In both cases loped in the scar) MGG ×400



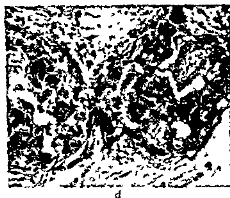
a



b



c

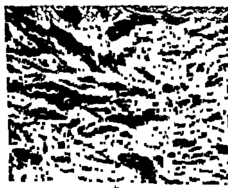


d

Fig 7 Cases in which the original histologic diagnoses of 'precancerous lesion' were re classified as intraductal carcinoma (group II in a—b and group III in c—d) and in which the cytologic reports from aspiration biopsy stated carcinoma a) Free carcinoma cells MGG ×400 b) Section from a tissue area reclassified as intraductal carcinoma Van Gieson ×250 c) Solid plug of carcinoma cells MGG ×400 d) Tissue section showing intraductal carcinoma Van Gieson ×250



a



b

Fig 8 Small cell carcinoma (Such cases were frequent among our cytologic 'false negatives') a) Aspiration biopsy smear MGG ×400 b) Tissue section Van Gieson ×250

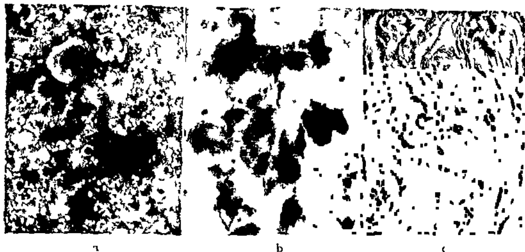


Fig 12 Ductal carcinoma with differentiation towards squamous epithelium a) Aspirate containing necrotic material and blue stained squamous carcinoma cells MGG ×400 b) Aspirate stained according to Papanicolaou ×400 c) Tissue section Cornified epithelium with pearl formation Van Gieson ×250

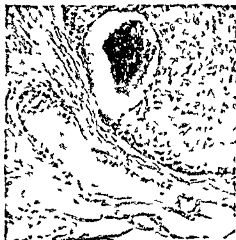


Fig 13 Tissue section from a mammary ductal carcinoma Needle track partly filled with blood Haematoxylin eosin × 50

Acta path microbiol scand Section B 79, 297-304, 1971

CELLULAR AND HUMORAL ANTIBODY PRODUCTION AGAINST SHEEP ERYTHROCYTES IN AKR MICE

I Effect of Antigen Dose and Treatment with 6-Mercaptopurin on the Primary Immune Response and on the Development of Immunological Memory

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The primary immune response and the immunological memory have been studied in four groups of AKR mice using the haemolytic plaque assay Two groups of mice, I and III, were given 4 10^6 and 4 10^8 SRBC, respectively, two other groups, II and IV, received additional 10 mg 6 mercaptopurin (6MP)/kg body weight for 7 days The primary 19S and 7S cellular antibody response in group III was 7-100 times greater than that in group I The 6MP treatment depressed the primary immune response The depression was greater in the mice stimulated with the lower antigen dose (group II) Ten days after the first stimulation all four groups of mice were given 4 10^8 SRBC Group I showed a marked immunological memory, whereas group III showed a secondary antibody response lower than the primary one Treatment with 6MP accentuated the difference It is discussed whether the reduction of immunological memory in this experiment is due to cellular exhaustion or inhibition by the antibody produced

The cellular reactions which take place after injection of antigen into experimental animals have been studied intensively after the development of the haemolytic plaque assay Stimulation of mice with sheep erythrocytes (SRBC) causes a proliferation of antibody forming cells After a lag period of the order of 20 hours the rise in plaque forming cells is exponential The resultant curve is determined by the rate of recruitment of antigen sensitive cells into antibody production, the rate of proliferation of cells already synthesizing antibody, and the life span of these cells The majority of antibody forming cells in mammals have a life-span of the order of 2-3 days while a minority might survive for several months (*Gowans* & *McGregor* 1965)

The antigen dose influences 1) the recruitment of antigen sensitive cells into antibody production and 2) the rate of proliferation of cells already synthesizing antibody (*Campbell* & *Kind* 1969, *Perkins et al* 1969, *Wigzell* 1969) Cellular proliferation can be depressed by various mitotic inhibitors (*Sahiar* & *Schwartz* 1965, *Sterzl* 1967 a) 6-mercaptopurin (6MP) was used in the present ex

Received 13 xi 70

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periments, it inhibits nucleic acid synthesis by blocking crucial reaction steps in the biosynthesis of adenylic acid and guanylic acid (*Mahler & Cordes* 1966)

Injection of antigen also generates immunological memory According to *Sterzl* (1962) and *Sercarz & Coons* (1962), an increase of the primary antigen dose may cause an exhaustion of memory cells and thereby a poor secondary antibody response However, the depressing effect of an exhausting primary antigen dose can be abolished partly by prolongation of the time interval between the two injections This probably occurs by a renewed recruitment of antigen sensitive cells, the so-called X cells, developing into memory cells (Y cells) (*Hege & Cole* 1966, *Sercarz & Byers* 1967) The phenomenon can also be explained, however, as a feedback inhibition by the antibodies synthesized during the primary immune response (*Wigzell* 1967 b)

The experiments reported here were carried out to find a correlation between the cellular and humoral antibody responses to two different doses of antigen Special attention was paid to the activity of persisting primary antibodies on the day of restimulation, and the influence of 6MP on both types of antibody response was studied

MATERIALS AND METHODS

Inbred AKR mice were obtained from the animal farm Hvidesten, of Statens Seruminstitut All animals were females 2–3 months of age and with body weight of about 20 g They were caged in groups of six and allowed food and water freely

Sheep red blood cells (SRBC) were supplied by Hvidesten The same sheep was bled for all experiments (blood taken sterily in Alsever's solution) The SRBC were washed three times with phosphate buffered saline M/15 pH 7 4 (PBS) and resuspended in PBS to give 4 10⁶ and 4 10⁸ SRBC per 0 2 ml This volume was injected intravenously into the tail vein of the mouse

6 mercaptopurin (6MP) was obtained from Merck 20 mg 6MP was dissolved in 2 ml 0 1 N NaOH and diluted 1 10 with saline to give 10 mg/kg body weight in 0 2 ml This was injected subcutaneously in the loose folds of the neck

Mice were bled from the retro-orbital sinus using a capillary pipette

The haemolytic plaque assay was carried out according to *Wortis et al* (1966) The developing antiserum was prepared in rabbits by hyperimmunization with rabbit red blood cells (RRBC) coated with mouse anti RRBC (doses of complex 4 10⁸ RRBC, injected intraperitoneally) The rabbit anti mouse immunoglobulin antiserum was tested according to *Wortis et al* (1966) A dilution of 1 25 was used with an inhibition coefficient of 0 705 and a developing coefficient of 1 165 (in this report direct plaques are called 19S and developed plaques 7S plaques)

Haemagglutination and haemolysis were carried out by the PVP method of *Stimpfling* (1961) using the microtitre equipment Diluting medium 1 5 per cent PVP in a balanced salt solution pH 6 9 (BSS), containing 0 14 g of $CaCl_2$ and 0 2 g $MgSO_4$ per litre A 1 per cent and a 2 per cent suspension of SRBC in BSS were used for haemagglutination and haemolysis respectively A guinea pig serum pool stored at —70°C thawed and diluted 1 10 in BSS immediately before use was employed as source of complement Incubation was made for 1 hour at 37°C in both determinations

Inactivation of antisera with 2 mercaptoethanol (2ME) was carried out according to *Uhr & Finkelstein* (1963)

Gel filtration was performed on Sephadex G200 0 5 ml of serum was separated on a K25/45 column (Pharmacia, Sweden) in BSS flow rate 15 ml per hour Fractions of 3 ml were collected in 10 ml tubes containing 0 1 ml 10 per cent BSA The protein content of the fractions was scored by means of an LKB Uvicord II, connected with an automatic writer (LKB type No 6810) The antibody and protein recovery was close to 100 per cent when 19S and 7S fractions were concentrated (by negative pressure dialysis) and tested serologically immediately after fractionation

Antiserum pools obtained from the four experimental groups of mice on day 10 following immunization were tested for inhibitory activity in vivo All antiserum pools under consideration (see Results) were titrated and tested for haemolytic and haemagglutinating antibodies They were then diluted to the same antibody concentration and 0 4 ml of antiserum dilution was injected intraperitoneally into AKR mice injected 2 days earlier with 4 10⁶ SRBC Differences in the percentage of inhibition were taken to be the relative binding activity of the antisera tested (*Walker & Siskind* 1968 *Moller* 1969 *Rubin* unpublished data)

Geometric means of plaque forming cells (PFC) per spleen of the different groups of mice were calculated according to *Wortis et al* (1966) The significance of differences between groups was evaluated by the students t test

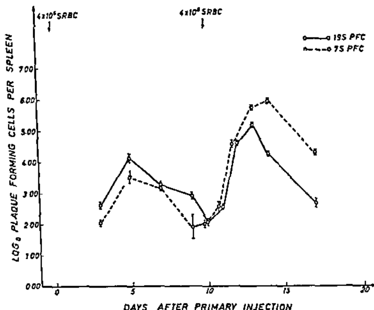


Fig 1 *Production of antibody against* SRBC *in* AKR *mice, measured* at the cellular level Vertical bars indicate standard error of mean For further explanation, see text

RESULTS

Forty AKR mice were divided into groups of 10, designated groups I to IV Groups I and II received a primary i v injection of $4 \cdot 10^6$ SRBC, groups III and IV $4 \cdot 10^8$ SRBC *Starting on the day of the primary stimulation*, the mice in groups II and IV were treated with 6MP, each mouse receiving 10 mg 6MP/kg body weight daily for 7 consecutive days (see Figs 1–4)

Figs 1 and 2 show the number of PFC, both 19S and 7S, in the two untreated groups of mice The mice stimulated with a low primary antigen dose show an extensive secondary antibody response after restimulation with a high antigen dose In contrast, the mice stimulated with a high primary antigen dose have a *poor secondary antibody response*, the level of PFC being even lower than after primary stimulation (see also Table 1)

The circulating haemolytic antibody was measured 10 and 17 days after primary stimulation *i e* before secondary stimulation and 7 days later The results are given in Table 1 The circulating antibodies followed the same pattern as the PFC

In the 6MP-treated groups, the mice injected *with the low primary antigen dose* showed a marked reduction in PFC after primary stimulation, compared to the similarly stimulated but untreated mice of group I (compare Figs 1 and 3) No such difference was noted between groups III and IV (compare Figs 2 and 4) This evaluation was based on the maximum values obtained 4–5 days after primary stimulation These values were as follows

Group I	$\log_{10}$ 19S PFC/spleen = 4 124±0 145
Group II	$\log_{10}$ 19S PFC/spleen = 3 140±0 195
Group III	$\log_{10}$ 19S PFC/spleen = 4 737±0 081
Group IV	$\log_{10}$ 19S PFC/spleen = 4 699±0 050

(± standard error of mean)

Group I : $\log_{10}$ 7S PFC/spleen = 3.515±0 204

Group II : $\log_{10}$ 7S PFC/spleen = 1 941±0 796

Group III: $\log_{10}$ 7S PFC/spleen = 4 653±0 105

Group IV: $\log_{10}$ 7S PFC/spleen = 4 570±0 098

The maximum 7S response on days 6-7 following primary stimulation with 4 10^8 SRBC was depressed in 6MP-treated mice as

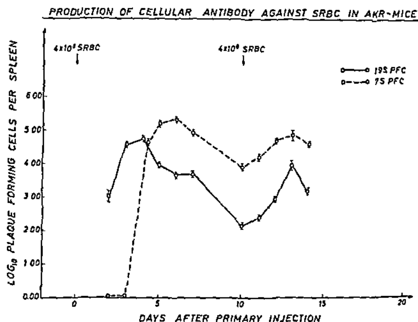


Fig 2 Text, see Fig 1

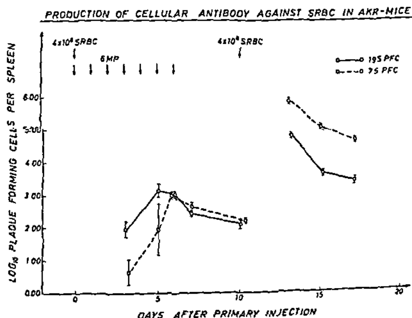


Fig 3 Text, see Fig 1

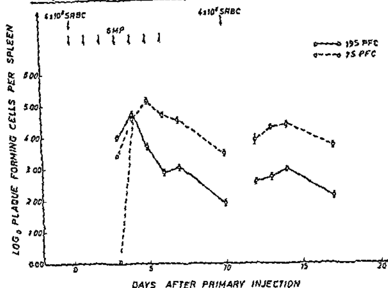


Fig 4 Text, see Fig 1 (+) Mice primarily stimulated with 4 10⁸ SRBC and treated with 6MP showed two different 7S PFC responses on day 3 after stimulation, one group of mice producing significant numbers of 7S PFC (four mice) and another group of mice producing no 7S PFC (six mice) This splitting up of the 7S PFC response on day 3 after stimulation is not seen in Fig 2, where the 7S response of all ten mice was negative

TABLE 1 *Antibody Production against Sheep Red Blood Cells in AKR mice Summary of Experimental Results*

Gr No	Antigen dose* Primary/secondary	6MP	Antibody class	Day 10 Log_{10} PFC per spleen	SE	Day 10/17 Log haem titre	Day 13 Log_{10} PFC per spleen	SE
I	$4\times10^8/4\times10^8$ SRBC	—	19S	2 063	0 123	4/8	5 122	0 050
			7S	2 007	0 123	<3/7	5 686	0 063
II	$4\times10^8/4\times10^8$ SRBC	+	19S	2 129	0 141	5/9	4 803	0 113
			7S	2 231	0 052	4/8	5 865	0 053
III	$4\times10^9/4\times10^8$ SRBC	—	19S	2 166	0 078	6/6	3 958	0 141
			7S	3 911	0 096	6/6	4 854	0 143
IV	$4\times10^9/4\times10^8$ SRBC	+	19S	1 912	0 101	8/6	2 725	0 091
			7S	3 481	0 092	6/7	4 270	0 054

* Primary stimulation day 0 Secondary stimulation = day 10

compared to 6MP untreated mice $\log_{10}$ 7S PFC/spleen = 4 690±0 086 *vs* $\log_{10}$ 7S PFC spleen = 5 324±0 063, respectively (see Figs 2 and 4)

Treatment with 6MP had no effect on the development of immunological memory in group II mice ('low" primary antigen dose), whereas the number of PFC after secondary stimulation was significantly lower in the treated group IV mice ('high" primary anti

TABLE 2 *Inhibition of 19S PFC Formation by Passive Antibody*

AKR mice groups	Inj with serum from AKR mice groups	*Titre at days 10 19S/7S	Dilut or undil	Log_{10} 19S PFC/spleen§		
				Normal	Serum treated	Inhib factor
V	I	4/<3	Undil	4 388	3 479	8 1
VI	II	5/ 4	Undil	4 388	3 477	8 1
VII	III	6/ 6	1 4	4 388	3 882	3 2
VIII	IV	8/ 6	1 4	4 388	4 081	2 0

* Haemolytic titre measured following gel filtration on Sephadex G 200
§ Measured day 5 after antigenic challenge

gen dose) than in the untreated "control' group III (compare Figs 2 and 4, see also Table 1)

The amounts of circulating haemolytic antibody 10 days after primary stimulation are shown in Table 1 In order to evaluate the relative binding capacity of the circulating antibodies, the following *in vivo* test was formulated

Serum from each of the four groups of mice taken on day 10 after primary stimulation was pooled By means of dilution, all four pools were given approximately the same haemolytic titre (see Tables 1 and 2) Each haemolytic serum pool was divided into 0 4 ml volumes and injected i p into groups of six AKR mice stimulated 2 days previously with 4 10^6 SRBC (i v) These mice, called groups V–VIII were tested for 19S PFC on day 5 after antigenic challenge and the results given as PFC/spleen were compared with the normal 19S PFC/spleen response in AKR mice The results of this experiment are shown in Table 2 Here it can be seen that serum pools from groups I and II ('low" primary antigen dose) caused a higher degree of inhibition than serum pools from groups III and IV ('high' primary antigen dose)

DISCUSSION

The present experiments were carried out in order to elucidate further the effect of cellular exhaustion and inhibitory activity of endogenously produced antibody on the development of immunological memory Using the mitotic inhibitor 6MP, answers were obtained to the pertinent questions 1) Is cell division an essential step in the generation of 19S and 7S PFC? 2) and in the development of memory? and 3) is there a different degree of dependence of division in the generation of 19S and 7S PFC?

Concerning the first question, the answer is cell division *is* an essential step in the generation of 19S and 7S PFC as treatment with 6MP causes that the primary immune response to SRBC is reduced to a greater extent when 4 10^6 SRBC is used as stimulus than when 4 10^5 SRBC is used *Sahiar & Schwartz* (1965) demonstrated that there was a selective inhibitory action of 6MP on 7S antibody synthesis in rabbits This is true in the present system using 4 10^5 SRBC (groups III and IV) Using 4 10^8 SRBC, the 7S PFC response though 1 day delayed is enhanced during the primary response (groups I and II) The higher degree of depression obtained with 6MP using the low antigen dose can be explained either by assuming 1) that the concentration of 6MP injected is insufficient to show a visible effect on the extensive cellular proliferation induced by the high (saturating) antigen dose, 2) that increased recruitment of antigen sensitive cells masks the effect of 6MP, or 3) by the findings obtained by *Möller & Greaves* (1970), namely that a low and a

high antigen dose preferentially stimulate different cell populations, thymus derived lymphocytes and bone marrow derived lymphocytes, respectively Which of these explanations is the true one is at present unknown

6MP has a greater effect on the production of 7S PFC in animals treated with the high antigen dose (group IV), especially later in the response This indicates that a pronounced 7S PFC outcome is more dependent on cell division than is 19S PFC production *Sterzl* & *Nordin* (1969) obtained results which seem to support the earlier hypothesis of *Sterzl* (1967 b) that 7S PFC is a product of a genetic shift in the dividing 19S memory cell compartment, *i e* 7S PFC needs a higher antigen stimulus to promote the proliferation and differentiation of their ancestor cells The present result seems to fit in with this hypothesis

The secondary immune response of groups I and II mice differed insignificantly, *i e* the 6MP dose used affected primarily the generation of 19S PFC, delayed the generation of 7S PFC, and had no effect on the development of 19S and 7S memory cells Groups III mice showed a poor secondary immune response, equal to or lower than the primary immune response (*Hege* & *Cole* 1966, *Sercarz* & *Byers* 1967, *Wigzell* 1967 a) *Sercarz* & *Byers* (1967) interpreted the results as an exhaustion of memory cells caused by the saturating primary antigen dose

Wigzell (1967 a) and *Möller* (1968) have shown that a reduced secondary antibody response may be caused by the inhibitory action of endogenously produced antibody As can be seen from Table 1, the concentration of haemolytic serum antibody on the day of secondary stimulation was 4–8 fold greater in groups III and IV animals compared to groups I and II This seems to favour the inhibitory action of endogenously produced antibody However, Table 2 shows that antibodies produced following stimulation with the low antigen dose are 3–4 times more efficient in binding antigen than antibodies produced following the high antigen dose, which should give approximately two antibody populations with similar antigen binding capacity (*Rubin* 1971)

6MP causes the development of a very poor secondary immune response (group IV) which is lower than the secondary response in group III mice This additional reduction cannot be explained entirely by an inhibition of endogenously produced antibody, as the serum antibodies in the two groups of mice were identical in antigen-binding capacity on the day of restimulation It is proposed that the simultaneous effect of exhaustion of cells and inhibition of endogenously produced antibody occur under these extreme conditions As reviewed by *Schwartz* (1965), the use of increasing antigen doses in co-operation with 6MP causes an increase in the instances of immunological unresponsiveness The high dose of SRBC in collaboration with 6MP causes an almost total recruitment of antigen sensitive cells, most of which develop into antibody producers, leaving a restricted number of cells as memory cells

It is evident that no clear-cut answer can be given to the question whether cellular exhaustion or inhibitory activity of endogenously produced antibody is the dominant factor in the regulation of antibody synthesis As the inhibitory activity of the antisera from the four experimental groups was similar on the day of restimulation, and as this seems to remain true with time after immunization (*Siskind* & *Benacerraf* 1969, *Rubin*, unpublished results) a high "saturating" antigen dose, in contrast to a lower antigen dose, probably causes a more or less irreversible change in the genom of the responding cells This can happen directly through a paralyzing effect of an overwhelmingly large amount of antigen compared with the number of antigen sensitive cells at disposal at the time of stimulation ("direct effect") or indirectly through a feedback mechanism when the circulating antibodies attain a critical level in amount and/or affinity ("indirect effect") These problems are now under study as they seem to have a bearing on the problems discussed in this paper

The author wishes to thank Dr *J Andersen* for reading the manuscript and for providing the opportunity of carrying out the work The excellent technical assistance of Mrs *I Skibshoj* is gratefully acknowledged My thanks are also due to Mr *F Valsted* who took care of the animals and performed the injections

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Acta path microbiol scand Section B 79, 305 313 1971

REGULATION OF ANTIBODY FORMATION AGAINST HUMAN SERUM ALBUMIN IN RABBITS

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Both the time interval between two antigenic stimuli and the size of the primary and secondary antigen doses have been varied in a study on antibody formation against human serum albumin (HSA) in rabbits It was found that the variation of the time interval be tween the primary and secondary stimulation did not significantly influence the amount of antibody produced in the secondary response This was true whatever the size of the primary dose of antigen However, when rabbits were stimulated with the same primary antigen dose, the amount of antibody produced in the secondary response was closely correlated with the dose of antigen used for restimulation The results are discussed in relation to present theories regarding the regulation on immunological memory

It has been suggested that maturation of the immune response depends on a cellular selec tion process based on the ability of antigen sensitive cells to bind antigen specifically to their surfaces (for a review, see *Siskind* & *Benacerraf* 1969) It is postulated that cells producing high affinity antibodies bind the antigen with high affinity, whereas cells producing low affinity antibody bind the antigen with low affinity Furthermore the binding of antigen to the cell is said to stimulate cell division and to increase antibody production Early in the immune response when the concentration of antigen is high, cells capable of forming high affinity antibodies as well as cells forming low affinity anti bodies are stimulated Later, when the con centration of antigen is low only cells capable of producing high affinity antibodies are stimulated As a result, the average binding energy of the late antibody population rises

The present study was carried out to establish the magnitude of the secondary antibody response to human serum albumin (HSA) in the rabbit as a function both of the time after the primary stimulus and of the secondary antigen dose The intention was to obtain an answer to three questions concerning secondary antibody production

1) Is there an optimal interval between primary and secondary stimulation?
2) Does the length of this interval depend on the primary antigen dose?
3) Does a direct proportionality exist between the secondary antigen dose and the magnitude of the secondary antibody response elicitated?

Studies relating to these questions have been carried out by agglutination methods

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(*Freund & Bonanto* 1942, *Barr & Glenny* 1945, *Fecsik et al* 1964), by precipitin titre methods (*Rubin 1971*), and by quantitative precipitation methods (*Dixon & Maurer* 1955, *Weigle* 1966, *Steiner & Eisen* 1967, *Walker & Siskind* 1968)

MATERIALS AND METHODS

Rabbits were supplied by the animal farm of Statens Seruminstitut They were all females, 3 months of age and of 2 5 kg body weight at the beginning of the experiments All injections were carried out intravenously (i v) into the ear vein The rabbits were bled from the ear (10 ml samples) or from the heart (40 ml samples) All blood samples were left to stand overnight at 4°C before isolation and inactivation (30 min at 56°C) of antisera, storage at —20°C

HSA was supplied by the Blood Fraction Department of the Statens Seruminstitut It had been isolated from human serum by a modified Cohn ethanol precipitation (fraction V)

Serology

Elimination The elimination either of 100 mg or of 10 mg of HSA from the blood circulation was measured by a precipitation reaction in agar plates by the method of Ouchterlony (15 ml 1 per cent Difco agar in phosphate buffered saline pH 7 38 (PBS) per plate), an anti HSA serum being applied to the central well and unknown serum samples in peripheral wells (well volumes in these agar plates were 0 1 ml) The method was standardized by applying the anti HSA serum to the central well and known HSA concentrations, including 100 mg and 10 mg to the peripheral wells and establishing the position of the precipitation band with each concentration The concentration of HSA in a serum was determined by reading the position of the precipitation band and comparing with the standard This technique was also used for titrations of HSA-containing sera (in 4 fold dilution steps) by determining the highest dilution showing a visible reaction with the anti serum in the central well The lower threshold of this method i e where a precipitation was just visible was 0 5 μg HSA/ml when tested against an anti HSA serum containing 10 mg of precipitating antibody per ml

Passive haemagglutination HSA was coupled to sheep red blood cells (SRBC) by means of bis diazotized benzidine (BDB) according to *Dresser & Wortis* (1967) The titration, in duplicate in 2 fold dilutions was performed with the microtitre equipment using 0 025 ml serum in each test The reaction was read as the last dilution step showing a + + reaction

Precipitin titre determination The precipitin titre reaction was performed in agar plates by the Ouchterlony method (see "Elimination) A 2 fold dilution series was applied to the peripheral wells and tested against HSA The precipitin titre was read as the last dilution step which could form a visible precipitation band with the HSA in the central well

Quantitative precipitation The total amount of specific, precipitating anti HSA in the serum was determined by this method To a number of tubes, each containing 0 5 ml of anti HSA serum 0 5 ml of a 2 fold HSA dilution series in PBS was added Following incubation for 1 hour at 37°C the reaction was completed after standing for 48 hours at 4°C After centrifugation (30 min at 3000 rpm) the supernatants were tested in Ouchterlony plates for content of non precipitated HSA and anti HSA, respectively The protein content of the washed precipitates was determined using the biuret method, standardized with a dilution series of HSA The total amount of precipitating anti HSA was calculated at equivalence by subtraction Very strong anti HSA sera (more than 5 mg/ml serum) had to be diluted before the test, since otherwise it was not possible to obtain a sharp equivalence point and thus total precipitation with a 2 fold dilution series of HSA did not occur (*Rubin*, unpublished)

Statistics Statistical calculations were carried out by *M Weis Bentzon*, actuary, the Biostatistical Department, Statens Seruminstitut, using variance analysis

RESULTS

In the present study on the formation of antibody to HSA in rabbits all sera were studied by the three above mentioned serological methods As complete correlation was found between, on the one hand, haemagglutination titres and precipitin titres and, on the other, quantitative precipitation values the results are expressed in terms of the quantitative precipitation values only The correlation between the two types of titre and the total quantity of precipitating anti-HSA is seen in Fig 1

Experiment 1

Thirty six rabbits were divided into two groups, in group I 24 which received 10 mg HSA i v and in group II 12 which received 10 mg HSA i v as the primary stimulus 30, 90 and 150 days later 8 rabbits in group

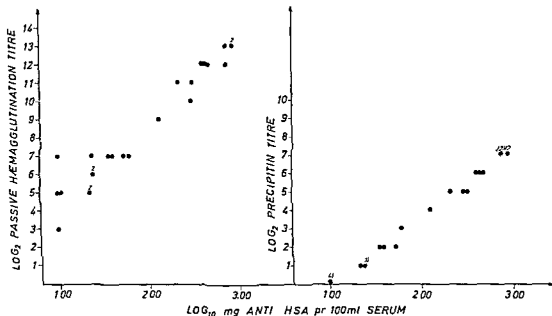


Fig 1 For explanation see Materials and methods

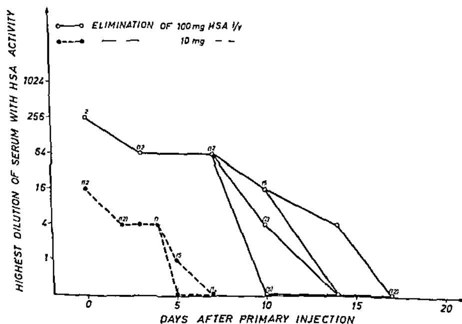


Fig 2 For explanation see Materials and methods

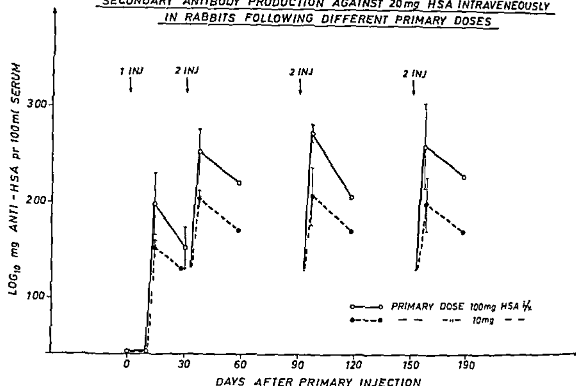


Fig 3 Vertical bars indicate one standard deviation For further explanation, see text, experiment I

I and 4 in group II received 20 mg HSA i v as a secondary stimulus Fig 2 illustrates the elimination of HSA after primary injection of 100 mg and 10 mg HSA respectively Both doses were followed by the triphasic elimination curve, characteristic of soluble protein antigens (*Dixon & Maurer* 1955, *Weigle & Dixon* 1957) 100 mg was eliminated from the circulation in 10 14 days and 10 mg in 5-7 days Thus, in the rabbit the elimination of 10 mg HSA takes about half the period required for elimination of 100 mg As is apparent from Fig 3, the primary antibody concentration was significantly higher in rabbits stimulated with 100 mg than in those stimulated with 10 mg HSA The peak value was attained in both cases 12 15 days after the primary injection The secondary antibody response on re-stimulation with 20 mg HSA after 30, 90, and 150 days, respectively, was not significantly different On the other hand, as is also evident from Fig 3, the total quantity of secondary anti HSA in rabbits injected primarily with 100 mg was found to differ significantly from that in rabbits injected with 10 mg HSA 30 90, as well as 150 days after injection The relative increase in antibody production due to the secondary injection was the same in rabbits challenged primarily with 100 mg and in those challenged with 10 mg HSA

Experiment 2

100 mg HSA as the primary stimulus and 20 mg as the secondary stimulus were the two fixed parameters in this experiment in which 40 rabbits were stimulated and re stimulated at the following intervals 30 60 90, 120 150, 210, and 270 days As may be seen from Fig 4, the magnitude of the secondary antibody response was independent of the time interval This confirms the findings from experiment 1 It must be pointed out however, that although no significant differences between the groups were found, possibly due to the relatively marked standard deviation there is a tendency to a reduced antibody response after a lapse of about 200 days

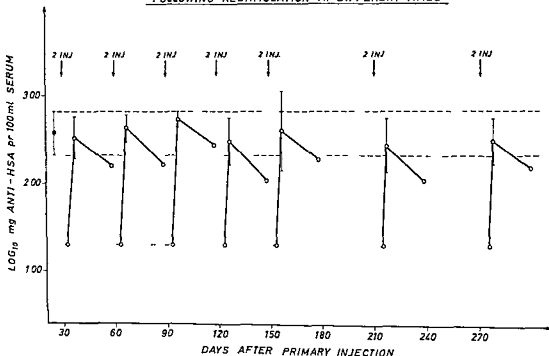


Fig 4 Vertical bars indicate one standard deviation, the calculated mean and one standard deviation for all groups of rabbits integrated are represented by the two horizontal dotted lines For further explanation see text, experiment 2

Experiment 3

Twenty eight rabbits were stimulated with 100 mg HSA i v and re stimulated 30 days later with the following doses of HSA 1, 5, 20, 40, and 60 mg The reason why these five secondary doses of antigen had been chosen was that the amount of anti HSA present in the serum 30 days after the primary stimulus was 30 90 mg anti-HSA per 100 ml (Table 1) It had been found that at equivalence this amount of antibody would bind from 5 to 20 mg HSA per rabbit, based on the assumption that about 125 ml plasma would be present in the circulation With the doses of antigen used for secondary stimulation it should be possible, teoretically, to obtain the following antigen-antibody combinations after injection 1 mg HSA representing a 5 fold antigen deficit, 5 20 mg HSA representing equivalence between the total amount of anti-HSA in the serum and the injected HSA and 40 60 mg HSA a 2- or 3 fold antigen excess

As is apparent from the experiment (Table 1), this does not apply in practice, or at least only with approximation In fact, these five doses used for secondary stimulation were treated in the rabbits as only two doses On the one hand, the antibody response was the same to 1 mg and 5 mg HSA and on the other, to 20 mg, 40 mg, and 60 mg HSA For this reason the secondary antibody response was interpreted statistically as belonging to two-dose ranges As is evident from Table 1 and Fig 5, the antibody response obtained using 20 60 mg HSA, *i e* theoretically antigen excess, gave the expected, previously obtained antibody response (experiment 1), significantly higher than the antibody response to 1-5 mg HSA, *i e* theoretically antibody excess

TABLE 1 *Effect on the Secondary Antibody Response* of Varying the Secondary Antigen Dose*

Group	Rabbit No	HSA (mg)	mg anti HSA/ml serum Day 30	Day 37§
I	2109	1	0.00	0.90
	2110	1	0.00	0.64
	2111	1	0.00	1.34
	2112	1	0.60	2.32
	2113	1	0.20	2.42
	2114	1	0.98	2.46
Average			0.33	1.48
II	2115	5	0.00	1.51
	2116	5	0.00	1.88
	2117	5	0.24	0.90
	2118	5	0.40	3.30
	2119	5	0.40	1.84
	2120	5	0.80	2.58
Average			0.42	1.85
III	2001	20	0.20	4.16
	2002	20	0.60	8.86
	2003	20	0.00	2.12
	2004	20	0.22	3.02
	3909	20	0.20	2.00
	3910	20	0.50	3.20
	3911	20	0.21	1.65
	3912	20	0.20	3.85
Average			0.28	3.33
IV	2005	40	0.00	1.30
	2006	40	0.36	4.64
	2007	40	0.24	4.48
	2008	40	0.40	8.72
Average			0.33	3.92
V	2009	60	0.54	7.52
	2010	60	0.00	0.00
	2011	60	0.21	7.52
	2012	60	0.21	3.18
Average			0.31	5.64
Average I + II			0.40	1.67
Average III + IV + V			0.29	4.18
Average I–V			0.33	—

*Rabbits were immunized with 100 mg HSA i v as a primary stimulus and rechallenged at 30 days

§The secondary antibody response was determined 7 days after the secondary stimulus

Statistics were performed with the variance analysis All figures were transformed to $\log_{10}$ units and geometric means of primary and secondary responses were determined with the following premises rabbits showing neither primary nor secondary response were excluded When calculating the means for rabbits 30 days after a primary injection of 100 mg HSA rabbits showing no primary response were excluded

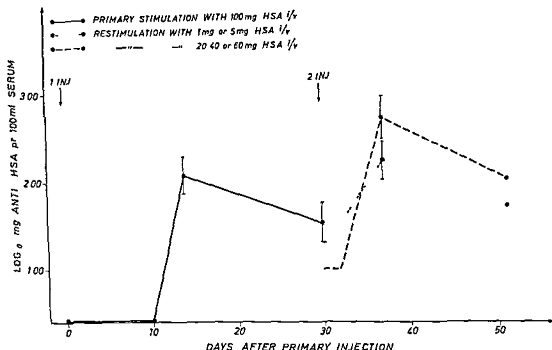


Fig 5 Vertical l nes indicate one standard deviation For further explanation see text experiment 3

DISCUSSION

In the present experiment, the following three factors were varied the primary antigen dose the secondary antigen dose and the time interval between the two antigenic stimuli The primary immune response to 10 and 100 mg HSA can be seen in Figs 2 and 3 As is apparent from Fig 2, the HSA reactive cells at the 100 mg antigen dose would be subjected to an antigenic stimulus over a period longer than that in the case with the 10 mg dose According to *Siskind & Benacerraf* (1969) rabbits stimulated with 100 mg HSA should produce an antibody population with a relatively lower avidity*) than rabbits stimulated with only 10 mg HSA The effect of the primary immune response to 10 mg HSA and 100 mg HSA on the secondary immune response to 20 mg HSA can be seen from Fig 3 Whatever the size of the priming dose of antigen the lack of an optimal time interval between primary and secondary stimulation is observed (see also Fig 4) *Weigle* (1966) obtained some what similar results using the antigens bovine serum albumin (BSA) and Keyhole Limpet haemocyanin (KLH) He found no reduction in the secondary antibody response to KLH in the course of about 2 years, whereas the secondary response to BSA was reduced by about 40 per cent in 7 20 months However, *Barr & Glenny* (1945) and *Fecsik et al* (1967) found an increasing amount of se condary antibody to be produced following an extended time interval *viz* from 30 to 90 days (antigen = diphtheria toxoid) and 10-40 days (antigen – diphtheria toxoid), respect ively Beyond 40 days *Fecsik et al* (1967) found no further increase in the secondary antibody response This discrepancy in the results referred to is perhaps caused by 1) the

* Affinity is the average binding constant in an antibody hapten system Avidity is the capacity of an antibody sample to form stable complexes with a macromolecular antigen

different antigens and 2) the different methods used *Barr & Glenny* (1945) and *Fecsik et al* (1967) used passive haemagglutination which detects 19S and 7S antibodies equally well, while *Weigle* (1966) and the present experiments made use of precipitin methods which detect 7S antibodies preferentially (*Wei & Stavitsky* 1967) Other workers (*Hege & Cole* 1966, *Wigzell* 1966, *Sercarz & Byers* 1967) found an optimal time interval of about 10 days for the 19S antibody response to sheep erythrocytes using a low/high antigen dose combination for primary and secondary stimulation The lack of an optimal time interval for the 7S antibody response can possibly be explained by the finding (*Allansmith et al* 1969) that IgG levels are under hereditary control, while the IgM levels are under environmental control (for a review see *Uhr & Möller* 1968)

As shown in Fig 5 and Table 1, a direct proportionality was found between the secondary antigen dose (in the range of 1-60 mg HSA) and the magnitude of the secondary response (*Freund & Bonanto* 1942) At least five factors seem to have an influence on the secondary immune response

1) The number of memory cells
2) The affinity of the antigen receptors on these cells
3) The amount of persisting antibody molecules in circulation
4) The affinity of persisting antibody molecules in circulation
5) The amount and nature of the antigen (see also *Siskind & Benacerraf* 1969)

It has been shown that immune complexes have decreasing immunogenicity as the amount of antibody per antigen molecule increases (*Bernardini et al* 1970) and that small amounts of antibody of high affinity enhance the immunogenicity of an antigen (*Walker & Siskind* 1968) Thus, both by extending the time interval and by increasing the secondary antigen dose antigen-antibody complexes of high immunogenicity are produced This is perhaps an alternative explanation of the lack of an optimal time interval described in this paper Whether the lower antibody response to the lower secondary antigen dose in experiment 3 is caused by a direct effect of antigen on the antigen sensitive cells (memory cells) or by an inhibitory effect of persisting antibodies on the complexing of antigen is at present unknown Unpublished results (*Rubin* 1971) have shown that a high amount of antibodies of low avidity and a low amount of antibodies of high avidity have the same regulatory effect on the secondary antibody response in rabbits

Our thanks are due to Dr *Joan Rhodes Madsen* for reading the manuscript and for helpful advice We also want to acknowledge the assistance of Mr *C Bergman* who took care of the animals and performed injections and bleedings

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Acta path microbiol scand Section B 79, 314–324, 1971

THE BURNS UNIT IN COPENHAGEN

10 *Antibiotic Sensitivity of* Staphylococcus aureus *Isolated from Burns*

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and the Institute of Medical Microbiology, University of Copenhagen

In the Burns Unit, Copenhagen, *Staphylococcus aureus* was isolated from 80 per cent of patients showing clinical infection in the burns. Most strains were resistant to penicillin streptomycin, tetracyclines, erythromycin, neomycin, and bacitracin. These multiresistant strains of *S. aureus* were found to occur with increasing frequency in extensive burns, the incidence increasing parallel with the duration of patient's stay in hospital and increasing duration of infection. The total incidence increased in the Unit in the course of the study period, and in 1968 multiresistant strains were isolated from 94 per cent of the patients infected with *S. aureus*. This endemic occurrence is explained by the use of neomycin and bacitracin for the local treatment of the burns. Erythromycin has never been used. Moreover, in the case of fusidic acid a relationship between the antibiotic consumption and the appearance of strains with reduced sensitivity was demonstrated. At admission, 30 per cent of the patients were found to harbour *S. aureus* in the nose, but only 2.5 per cent were subsequently infected by their own nasal staphylococci, and in most cases these staphylococci were later overgrown by the multiresistant strains.

In previous publications from the Burns Unit, Copenhagen the lay-out of the Unit has been reported together with its therapeutic principles (*Sørensen & Thomsen* 1968 a), the composition of the series of patients (*Thomsen & Sørensen* 1968), and the results of surgical treatment (*Sørensen & Thomsen* 1968 b). The infection problems have been elucidated by relating the occurrence of clinical infection to the patient material (*Thomsen* 1970 b), and the bacteriological findings have been described by way of an analysis of the distribution of various bacteria in the various categories of patients (*Thomsen* 1970 c). The composition of the bacterial flora proved to be dependent upon the age of the patient and the extent and depth of the burn. Moreover, since the bacterial flora varied during the patient's stay in hospital the result was assessed on the basis of the total occurrence of various bacteria in the individual patient. The predominant microorganism proved to be *Staphylococcus aureus* which was isolated from 80 per cent of the patients who showed clinical signs of infection in the burn. Among patients who did not exhibit any signs of infection, but who were found to have bacteria in the burns only 35 per cent harboured *S. aureus*. These patients are designated merely as bacterially contaminated.

As reported in previous publications, swabs for culture were taken from the nose, throat, and burned areas of all patients at admission (*Thomsen* 1970 a). Practically all patients were treated by exposure (*i.e.* without dressing). This rendered it possible to make thorough studies of the infection problems and to take swabs for culture regularly from the burns. The technique used in swabbing and

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the results of the cultures have previously been described in detail (*Thomsen* 1970 a and c) Owing to the isolated location of the Unit, to the fact that it admits only patients with burns and that it has its own staff, particular interest has been devoted to the epidemiological problems right from its start in November 1961 Since *S aureus* has proved to be the predominant bacterium, this organism has been the subject of the most thorough studies of the sensitivity pattern to antibiotics Studies on the changes in this pattern will be described below

PREVIOUS PUBLICATIONS

Rivera et al (1956) demonstrated that *S aureus* isolated from burns during the period 1950–54 showed decreasing sensitivity to penicillin, streptomycin and tetracycline Only a few strains were resistant to chloramphenicol, and nearly all were fully sensitive to neomycin

Körlof (1956) was able to relate this reduced sensitivity to the use of antibiotics In 1958 it was demonstrated by *Moncrief* & *Rivera* that *S aureus* strains from burns were sensitive to penicillin in only 4 per cent of all cases *S aureus* soon developed a reduced sensitivity to erythromycin but the isolated strains were still fully sensitive to neomycin and bacitracin

Haynes et al (1960) found that the percentage of patients with burns from whom *S aureus* could be isolated had increased from 30 to 89 in the period from 1951 to 1959 They ascribed this development to the increased use of antibiotics and supplied evidence of changes in the infection rate in conformity with the consumption of antibiotics However, half the strains isolated in 1959 were resistant to erythromycin, although this antibiotic had been used but sparsely Bacitracin and neomycin were used topically but any significant resistance to these agents had not developed

Birke et al (1960) found *S aureus* to be sensitive to penicillin in 10–20 per cent of their cases, to streptomycin in 20–30 per cent, and to tetracyclines in 50 60 per cent Nearly all strains were sensitive to erythromycin and chloramphenicol but the sensitivity decreased after systemic as well as local use of antibiotics

Kefalides et al (1964) found *S aureus* isolated from burns in children treated with antibiotics to be sensitive to tetracyclines in 21 per cent, to chloramphenicol in 37 per cent, to erythromycin in 60 per cent, to kanamycin in 74 per cent, to novobiocin in 80 per cent, and to bacitracin in 97 per cent

As a consequence of this development in antibiotic sensitivity, resistant strains soon began to predominate the infection findings in burns centres *Clarkson* & *Greenwell* (1958) demonstrated a "resident staphylococcus' resistant to most antibiotics (penicillin, streptomycin and tetracyclines), and *Wickman* (1958) described an endemic which had occurred in a burns unit and was caused by *S aureus* resistant to erythromycin After the treatment of two cases of septicaemia with erythromycin, resistant *S aureus* was isolated from another 5 patients, from the noses of members of the staff, from utensils, and from the air in the unit throughout 5 months after the treatment had been discontinued During a clinical trial *Lowbury et al* (1962) showed the emergence of *S aureus* resistant to Fucidin in 7 patients not treated by the latter and *Lowbury et al* (1964) demonstrated dissemination of *S aureus* resistant to neomycin after only one month s use of polymyxin neomycin bacitracin spray for the local treatment of burns

MacMillan (1967) found that 94 per cent of patients with burns harboured *S aureus*, 83 per cent of which were of a phage type characteristic of the unit and resistant to penicillin, streptomycin, and tetracycline Only 25 per cent of the strains were resistant to erythromycin, 12 per cent to neomycin, and 10 per cent to bacitracin

In 1967 *Shallard* & *O Connor* reported the spread of a multiresistant *S aureus* in a children's burns ward Most of the isolated strains were resistant to erythromycin and half of them also to neomycin Especially the latter

21*

were more apt than other strains to spread in the burns, but did not always entail clinical infection

MATERIAL

Among the patients in the Burns Unit, Copenhagen, who harboured bacteria on the burned skin already at admission, 15 per cent had *S aureus*, viz 50 out of 1498 acute cases (3 3 per cent) During the stay in the Unit, *S aureus* was demonstrated in an increasing number of patients After a stay of less than 15 days it was found in 60 per cent of the patients designated as clinically infected and in 30 per cent of those designated merely as bacterially contaminated Among patients who stayed for more than 30 days in the Unit, *S aureus* was present in 88 per cent of the infected and in 68 per cent of the contaminated ones (*Thomsen* 1970 c)

A study of the sensitivity of *S aureus* in patients in the present series revealed that a number of patients harboured *S aureus* of relatively high antibiotic sensitivity shortly after admission Later, this strain would disappear entirely or co-exist with a strain showing a considerably reduced sensitivity, as a rule the one which predominates in the Unit and is resistant to penicillin, streptomycin, tetracyclines, erythromycin neomycin and bacitracin Since it seemed to me that it might be of interest to investigate how many patients gradually acquired this resident *S aureus* I analysed the results on the basis of such strain of *S aureus* as had been isolated from each patient and found to be resistant to the largest number of antibiotics

Therefore, the sensitivity patterns are related to the total patient material by listing each patient as infected, or contaminated, with the strain which exhibited reduced sensitivity to the largest number of antibiotics On the other hand the incidence is not calculated on the basis of the total number of cultures Indeed, this would give a distorted picture of the bacterial findings, as all the patients did not have an equal number of cultures done

METHODS

The strains of *S aureus* isolated in the Unit since it opened in November 1961 and until the middle of 1965, were studied in the Institute of Medical Microbiology, University of Copenhagen The strains were stored as stab cultures in agar medium and again studied for sensitivity to antibiotics by the present author in 1968 The technique has been described by *Jensen* & *Kjær* (1947), by *Dragsted* & *Erichsen* (1953), and by *Eriksen* & *Erichsen* (1964) Those strains which showed reduced sensitivity to erythromycin were also tested with lincomycin oleandomycin and spiramycin

From the middle of 1965 to the end of 1968 the *S aureus* strains isolated in the Burns Unit were studied in the Bacteriological Department of Kommunehospitalet (the Municipal Hospital) The technique has been described by *Thomsen* (1967), but was used without pre diffusion

Furthermore the multiresistant strains isolated during the latter half of 1968 were studied by the present author against the same antibiotics as those used in the years 1961 65

TABLE I *The Burns Unit in Copenhagen 1961–68 Occurrence of Resistance to Various Antibiotics in* S aureus *Isolated from Burns*

	1962	1963	1964	1965	1966	1967	1968	Total
No of pts with *S aureus*	69	98	60	81	62	83	109	561
	Percentage of patients harbouring *S aureus* resistant to							
Penicillin	87	92	82	80	77	93	92	87
Streptomycin	71	75	52	54	50	75	73	66
Tetracyclines	36	70	47	51	47	74	73	59
Chloramphenicol	6	1	7	10	21	0	3	6
Erythromycin	32	65	42	47	29	70	70	53
Fusidic acid	0	13	8	0	0	0	0	3
Methicillin	0	0	0	0	2	4	1	1
Neomycin	26	52	37	(19)	—	—		?
Novobiocin	0	0	0	0	—	—		?
Bacitracin	26	52	33	(19)	—	—		?
Percentage sensitive to all antibiotics	13	8	18	20	23	7	8	13

RESULTS

Table 1 lists the annual number of patients who harboured *S aureus* in their burns as well as the percental occurrence of resistance to the studied antibiotics At the bottom the table gives the percentage of patients who showed throughout their stay only *S aureus* sensitive to all antibiotics The percentages indicate the annual proport on of patients who harboured *S aureus* resistant to the anti biotic concerned although these patients are listed only once in the table for each anti biotic even though they may have housed several strains with different resistance pat terns

Right from the time that the Unit opened the frequency of resistance to penicillin has been high and has varied but little The same applies to streptomycin However in 1964–66 there was an increased sensitivity to strep tomycin less so to penicillin The same changes in sensitivity were found to apply to tetracyclines and erythromycin These find ings are illustrated in Figure 1 together with the annual variations in the incidence of clinical infection There was a fall in incid

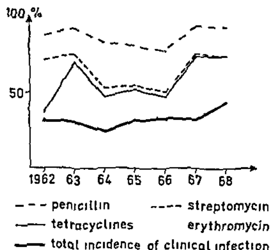
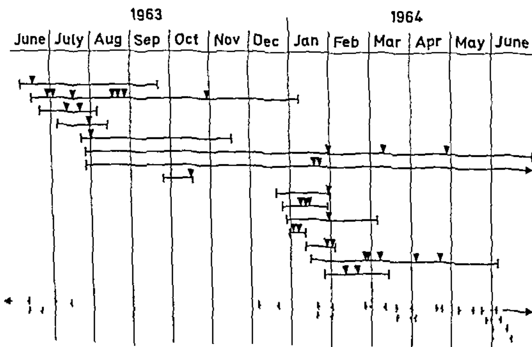


Fig 1 Inc dence of resistance to ant b ot cs in *S a eus* isolated from all pat ents adm tted 1961 68



F g 2 Isolat on of fus d c ac d res stant strains of *S aureus* in a cl n cal trial of fucidin 1963 1964 The sol d l nes ind cate the durat on of hosp tal zat on of the individual pat ents triangles the date of isolat on and the broken l nes patients treated with fuc d n

4 pts were treated in May 1963 and 3 n July 1964

ence only in 1964 and an increase in 1968 and hence, this cannot explain the observed changes in sensitivity to antibiotics

The occurrence of a few strains resistant to fusidic acid in 1963 and 1964 must be viewed on the background of the experimental administration of this antibiotic to a few patients during these years *(Sorensen et al* 1966) This entailed minor endemics of fusidic acid resistant *S aureus* (Figure 2) *S aureus* has not been studied for sensitivity to neomycin, novobiocin, and bacitracin during the last 3 years, and the same applies to some of the strains from 1965 The percentages obtained during this year are therefore stated in parentheses

Investigation of the corresponding percentages for patients treated with penicillin (mainly patients with large burns who, from the time of admission received penicillin prophylaxis) shows the same relative distribution of the sensitivity pattern except that all percentages are higher Part of the explanation is that patients who receive penicillin prophylaxis have extensive burns and therefore stay for longer periods in hospital Thus, the risk of contamination with multiresistant *S aureus* will be increased

The occurrence of resistance to a number of antibiotics in patients treated thereby is shown in Table 2 Except for the patients treated with Fucidin, all these patients received also penicillin, either simultaneously with or before treatment with other antibiotics Any definite influence upon the sensitivity of the isolated strains cannot be traced except in the two patients who developed methicillinresistant *S aureus* after treatment with this agent It is particularly striking that none of the 22 patients treated with Fucidin developed *S aureus* with a reduced sensitivity to this drug while such strains were isolated from other patients (Fig 2) Possibly, the reason may be that most of the patients who received experimental Fucidin therapy had small burns which healed without infection Therefore, the demonstration of *S aureus* may have failed In addition these patients may have excreted fucidin resistant strains with the stools which were not investigated On the other hand, all patients treated with polymyxin and with oxacillin were severely infected, therefore, they stayed longer in the Unit and thus ran a greater risk of contamination The four patients treated with chloramphenicol had received this anti

TABLE 2 *The Burns Unit in Copenhagen 1961–68 Resistance Patterns of* S aureus *Isolated from Patients Treated with Various Antibiotics*

		Percentage of patients harbouring *S aureus* resistant to							
		Penicilin	Streptomycin	Tetracyclines	Chloramphenicol	Erythromycin	Fusidic acid	Methicillin	No of pts
Treated with	Streptomycin	100	78	65	10	45	7	3	31
	Tetracyclines	100	90	80	0	80	30	0	10
	Polymyxin B	100	98	98	13	87	15	3	39
	Fusidic acid	100	91	91	9	87	0	0	22
	Oxacillin	100	100	100	20	100	33	0	15
	Chloramphenicol	100	100	100	50	100	25	0	4
	Methicillin	100	100	100	31	92	0	15	13
	Gentamycin	100	100	100	14	100	0	0	7
	Sulfamylon	100	100	100	0	100	0	0	6
	Total number treated with antibiotics	100	92	89	14	81	12	3	147

TABLE 3 *The Burns Unit in Copenhagen 1961–68 Number of Patients Treated with Various Antibiotics*

Prophylaxis		Therapy	
Penicillin	309	Penicillin	235
Penicillin + streptomycin	18	Streptomycin	31
Oxacillin or fusidic acid	49*	Sulpha drugs	12
		Polymyxin B	42
		Tetracyclines	13
		Oxacillin	10
		Fusidic acid	7
		Chloramphenicol	5
		Methicillin	14
		Gentamicin	7
		Sulfamylon (topically)	9
		Total number of pts treated with antibiotics	256

* The experimental series mentioned in the text

biotic before they were transferred to the Burns Unit The same applies to most patients treated with streptomycin and tetracyclines

The total number of patients who received antibiotic medication is given in Table 3 In addition to the data stated in the table, five patients received vancomycin, ampicillin, cephaloridine, carbenicillin, and colistin Out of the 31 patients treated with streptomycin 12 were treated in 1962 Methicillin was not used until 1966, gentamicin and sulfamylon not until 1968

The listed antibiotics were administered

TABLE 4 *The Burns Unit in Copenhagen 1961–68 Distribution of Various Resistance Patterns in* S aureus *Isolated from Patients with Clinical Infection among Those Admitted as Acute Cases*

	Total percentage with clinical infection	Percentage harbouring *S aureus* in burns	Sensitive	Per cent with resistance			
				P	PS	PST	PSTE*
	%	%	%	%	%	%	%
1962	28	83	13	15	33	4	35
1963	26	90	2	9	4	2	83
1964	21	68	11	26	7	0	56
1965	24	71	12	17	3	3	65
1966	28	67	23	23	8	19	27
1967	33	85	4	7	2	0	87
1968	45	85	2	4	0	0	94
Total	28	80	8	13	9	3	67

* P = Penicillin
S = Streptomycin
T = Tetracyclines
E = Erythromycin

TABLE 5 *The Burns Unit in Copenhagen 1961–68 Distribution of Various Resistance Patterns in* S aureus *Isolated from Patients without Clinical Infection among Those Admitted as Acute Cases*

	Total percentage contaminated, without infect	Percentage harbouring *S aureus* in burns	Sensitive	Per cent with resistance			
				P	PS	PST	PSTF
	%	%	%	%	%	%	%
1962	33	32	40	40	5	10	5
1963	23	41	14	38	7	7	34
1964	29	37	29	21	7	7	36
1965	18	40	33	20	7	27	13
1966	26	23	20	60	0	0	20
1967	25	31	7	29	0	29	35
1968	28	40	11	22	0	6	61
Total	24	35	22	32	4	12	30

systemically Only sulfamylon was applied topically Besides, ever since the Unit opened we have used spray with polymyxin B neomycin, and bacitracin for topical treatment Furthermore since 1964 nitrofurazone in carbowax impregnated in gauze mesh has been used The indications for local treatment by these agents have been discussed previously (*Sorensen & Thomsen* 1968 a)

To obtain a pure picture of the epidemiological status in the Unit I collected in Table 4, all acute cases in which clinical infection developed The patients concerned had not been in contact with any other hospital environment on account of their present burns (*Thomsen* 1970 a) The first column gives the percentage of acute cases with clinical signs of infection Minor year by year variations have occurred but a clearly divergent rate of infection is not seen until 1968 (*Thomsen* 1970 b) Moreover the percentage of patients infected with *S aureus* has been almost parallel with the annual rate of infection On the other hand the occurrence of resistant strains though distinctly increasing did not increase in step with the total infection rate

Table 5 sets out the corresponding percentage occurrence of resistance patterns in patients who did not exhibit clinical signs of infection, but whose burns nevertheless were contaminated by bacteria (*Thomsen* 1970 a) The incidence of merely contamination is seen to vary less than that of clinical infection in the former category of patients the frequency of occurrence of *S aureus* was only half that seen in infected patients A much larger number of sensitive strains occurred and most of the others were resistant only to penicillin 1968 constitutes an exception in that strains resistant to PSTE predominated also in this group of patients

Among acute cases *S aureus* was demonstrated for the first time in the burns of 34 per cent of the patients in the course of their first week in hospital and in 35 per cent of the patients more than 15 days after admission (Table 6) The sensitivity of the strains isolated on this first occasion showed characteristic changes at different times In burns infected at an early stage 26 per cent of the organisms were sensitive to all antibiotics At the end of one week all were resistant to one or more antibiotics and in the majority, the predominant pattern was PSTE

This distribution is apparent also when comparing the *S aureus* strain with reduced sensitivity to the largest number of antibiotics

TABLE 6 *The Burns Unit in Copenhagen 1961–68 Sensitivity to Antibiotics of S aureus in Infected Burns Isolated at Different Times after Admission*

S aureus isolated first on	Per cent of pts	Sensitive %	Resistance pattern P, PS, PST %	PSTE %
3rd day	13	26	37	37
5th day	8	9	35	56
7th day	13	0	34	66
10th day	18	15	29	56
15th day	13	15	32	53
> 15th day	35	3	29	68
No of pts	302	29	95	178

isolated from patients who had been hospitalized for varying lengths of time (Table 7) Among patients with clinical infection as well as among those showing merely contamination *S aureus* occurred in an increasing number of patients the longer they had been in hospital Moreover, the frequency of erythromycin resistant strains increased in both categories, but were still almost twice as common in infected as in merely contaminated burns At the same time, the sensitive strains decreased in frequency, most markedly in the contaminated burns

These changes in the sensitivity of S *aureus* are even more pronounced when patients with clinical infection of increasing duration are considered (Fig 3) Below zero line is plotted that percentage of patients who harboured sensitive strains during the period concerned, above zero line the percentage who harboured strains with reduced sensitivity Evidently, the erythromycin resistant strains possess an ability to oust other strains with a greater sensitivity to antibiotics This is no doubt related to the long lasting, extensive use of polymyxin B, neomycin, and bacitracin for the local treatment of patients with clinical infection On the other hand, erythromycin has never been used in the Burns Unit

Among the acute cases who later developed clinical infection, 15 harboured *S aureus* on the burned skin already at the time of admission In 7 the strains were sensitive to all antibiotics, in 5 resistant to penicillin, and in the others to PS, PST, and PSTE, respectively Eight of these patients subsequently exhibited *S aureus* of a different sensitivity (1 P, 1 PS, 1 PST and 5 PSTE)

TABLE 7 *The Burns Unit in Copenhagen 1961–68 Occurrence of Various Resistance Patterns of* S aureus *Isolated from Patients with Clinical Infection and Merely Contaminated Patients after Different Durations of Stays in Hospital*

Duration of stay	Per cent clinically infected with *S aureus*	Per cent sensitive	P	PSTE	Per cent merely contamin with *S aureus*	Per cent sensitive	P	PSTE
< 10 days	59	17	16	37	29	35	48	17
10 15 days	62	19	50	31	35	28	44	28
16–20 days	77	12	55	33	45	25	56	19
21 30 days	83	16	23	61	55	18	43	39
> 30 days	88	6	23	71	68	8	46	46

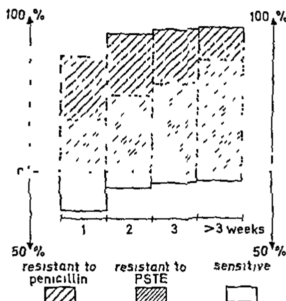


Fig 3 Frequency of *S aureus* of different antibiotic sensitivity depending upon duration of infection

Forty-four patients with clinical infection had first one strain of *S aureus* and later another one with altered sensitivity 32 acquired a strain resistant to PSTE, 5 to PST, 4 to PS, and 3 to penicillin only Prior to this change, 29 of the patients had been treated with penicillin, 3 with penicillin and streptomycin, and 2 with penicillin and tetracyclines

Among the patients who became contaminated with *S aureus* while in hospital, but did not exhibit clinical signs of infection 12 had sensitive strains, 27 penicillin-resistant ones—9 of the pattern PST and 32 of the pattern PSTE. From 8 of these patients another strain was isolated later, 5 PSTE, 1 PST, and 2 PS

In nearly all cases where *S aureus* of an altered sensitivity was demonstrated later, the organism was of a phage type different from the first one

From November 1961 until the end of 1968, a total of 1676 patients were admitted to the Burns Unit without having previously been admitted elsewhere At the time of admission of patients, *S aureus* was isolated from the nares in 489 cases, from the throats in 216 cases, and from both sites in 98 cases According to antibiotic sensitivity and phage

TABLE 8 *The Burns Unit in Copenhagen 1961–68 Sensitivity to Antibiotics of the* S aureus *Isolated from the Nose and Throat at Admission (All Patients)*

		S aureus in the nose	*S aureus* in the throat	Same strain in nose/throat and burn	*Clinically infected*	Merely contaminated	*S aureus* only in burn on adm
		%	%	%	%	%	%
Primarily admitted	Sensit	38	48	32	35	28	38
	P-resist	57	45	56	53	60	38
	PS	2	4	6	5	8	16
	PST	2	2	6	7	4	8
	Per cent of adm pts	30	13	4	63	37	52
Previously hospitalized	Sensit	13	10	9	11	0	0
	P-resist	49	56	31	30	38	40
	PS	5	6	7	8	0	0
	PST	17	10	24	22	38	20
	PSTC	2	4	5	3	12	20
	PSTE	14	14	24	27	12	20
	Per cent of adm pts	32	17	15	82	18	63

type the same strain was demonstrable in the burns in 68 patients among these patients 43 developed clinical infection In 13 patients *S aureus* of the same type was found in the burns only at admission The sensitivity to antibiotics of the *S aureus* isolated at the time of admission from the nares and throat is shown in Table 8

Out of 305 patients who were transferred from other hospitals or previously had been admitted to the Burns Unit 97 harboured *S aureus* in the nose 51 in the throat and 35 in both sites In 45 patients the same strain colonized the burns and among patients in this group 37 developed clinical infection during their stay in the Unit Sensitive strains were rare in these patients Resistance to penicillin was found to occur with the same frequency as in patients who had not previously been admitted and multiresistant *S aureus* occurred in almost half the patients

DISCUSSION

The endemic presence of erythromycin resistant *S aureus* in the Burns Unit Copenhagen is demonstrable in the individual patients as well as in the Unit as a whole It should presumably be considered in the light of the extensive use of neomycin and bacitracin for the local treatment of the burns Erythromycin has never been used but practically all isolated strains of *S aureus* which showed reduced sensitivity to this antibiotic were also resistant to neomycin and bacitracin The selection of multiresistant strains which have developed on these grounds were demonstrable a short time after the Unit opened

Jevons et al (1966) also found neomycin bacitracin resistance to be contributory to the rapid spread of these *S aureus* strains in a hospital environment On the other hand *Gillespie et al* (1961) reduced the frequency of cross infection by multiresistant *S aureus* by treating major wounds locally by a spray of polymyxin B neomycin and bacitracin

Lowbury (1964) has demonstrated that the number of patients in whom *S aureus* resistant to neomycin developed would be larger among those treated with the latter than among patients who merely became contaminated with the neomycin resistant strains although the drug had not been locally applied After the treatment was discontinued the resistance to neomycin in *S aureus* decreased although it was demonstrable in some patients several months after discharge

The large number of bacteria in patients with burns increases the risk of spread from these patients (*Rountree* 1963), and so does presumably the exposure treatment practised in the Burns Unit Copenhagen (*Penikett et al* 1958 and *Barclay & Dexter* 1968)

It is remarkable that so few patients in the Burns Unit Copenhagen became infected with their own nasal staphylococci This indicates that auto infection is rare, and that most infections are cross infections with the named multiresistant *S aureus*

The explanation of the comparatively low frequency of nasal carriers among previously hospitalized patients in the present series is presumably that most of the patients who had been transferred had been in hospital only for a few days prior to their transfer and therefore they had not yet become nasal carriers of hospital staphylococci Most of the patients who previously had been admitted to the Burns Unit had been discharged 6–12 months before they were re admitted for further plastic surgery As a result they had resumed their normal carrier rate (*Siboni* 1960) However the frequency of multiresistant *S aureus* in the nose is higher among previously hospitalized than among those admitted for the first time (*Noble et al* 1964)

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THE BURNS UNIT IN COPENHAGEN

II *Phage Types of* Staphylococcus aureus *and the Relation to Antibiotic Sensitivity*

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In the Burns Unit, Copenhagen, *Staphylococcus aureus* was demonstrated in 80 per cent of burns which showed clinical infection All strains were phage typed The strains of the complex 52, 52A, 80, 81 decreased in frequency in the course of 1962 At the same time, there was an increase in the occurrence of the complex 84, 85, 6557, to which, in 1967, 80 per cent of the isolated *S aureus* strains belonged 93 per cent of these strains were resistant to penicillin streptomycin, tetracyclines, erythromycin, neomycin and bacitracin and were found to occur at increasing frequency in extensive burns, in cases of prolonged infection, and in patients showing a poor take of skin grafts Owing to this endemic occurrence, the material does not permit conclusions to be drawn concerning virulence, and distribution of the phage types demonstrated was the same as that in other hospital infections The spread of type 84/85/6557 was followed The sensitivity of these strains to erythromycin neomycin, and bacitracin was studied by the tube dilution method, Gradual transitions to resistance or dissociated resistance could not be demonstrated The resistance to erythromycin was of the inducible form, but an increasing resistance to oleandomycin developed The possibility that this strain may have arisen from 83A by lysogenic conversion is discussed

In a study of the bacteriological flora in the Burns Unit, Copenhagen *Staphylococcus aureus* was found to be the predominant bacterium in the burns, being isolated from 80 per cent of the patients with clinically infected burns (*Thomsen* 1970 b) Investigation of the antibiotic sensitivity of the isolated *S aureus* showed that strains resistant to penicillin streptomycin, tetracyclines, and erythromycin gradually predominated in the individual patients as well as in the entire Unit (*Thomsen* 1971)

Received 9 x 70

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A „resident staphylococcus" *in a burns unit* was mentioned for the first time by *Clarkson & Greenwell* (1958), and minor endemics of erythromycin resistant *S aureus* (*Wickman* 1958) and neomycin resistant *S aureus* (*Lowbury et al* 1964) have been reported

Decoulx et al (1965) found that on the 15th day after admission to a burns unit 78 per cent of the patients harboured in their burns *S aureus* of a phage type which had been demonstrated at admission in only 7 per cent

In a series of patients with burns treated during the period 1964–65, *MacMillan* (1967) found that 94 per cent harboured *S aureus* which in 83 per cent was of a phage type characteristic of the unit

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THE BURNS UNIT IN COPENHAGEN

II *Phage Types of* Staphylococcus aureus *and the Relation to Antibiotic Sensitivity*

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In the Burns Unit, Copenhagen *Staphylococcus aureus* was demonstrated in 80 per cent of burns which showed clinical infection All strains were phage typed The strains of the complex 52, 52A 80, 81 decreased in frequency in the course of 1962 At the same time, there was an increase in the occurrence of the complex 84, 85, 6557, to which, in 1967, 80 per cent of the isolated *S aureus* strains belonged 93 per cent of these strains were resistant to penicillin streptomycin, tetracyclines, erythromycin neomycin, and bacitracin and were found to occur at increasing frequency in extensive burns in cases of prolonged infection, and in patients showing a poor take of skin grafts Owing to this endemic occurrence, the material does not permit conclusions to be drawn concerning virulence, and distribution of the phage types demonstrated was the same as that in other hospital infections The spread of type 84/85/6557 was followed The sensitivity of these strains to erythromycin, neomycin, and bacitracin was studied by the tube dilution method Gradual transitions to resistance or dissociated resistance could not be demonstrated The resistance to erythromycin was of the inducible form, but an increasing resistance to oleandomycin developed The possibility that this strain may have arisen from 83A by lysogenic conversion is discussed

In a study of the bacteriological flora in the Burns Unit, Copenhagen, *Staphylococcus aureus* was found to be the predominant bacterium in the burns, being isolated from 80 per cent of the patients with clinically infected burns (*Thomsen* 1970 b) Investigation of the antibiotic sensitivity of the isolated *S aureus* showed that strains resistant to penicillin streptomycin, tetracyclines, and erythromycin gradually predominated in the individual patients as well as in the entire Unit (*Thomsen* 1971)

Received 9 x.70

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A „resident staphylococcus" in a burns unit was mentioned for the first time by *Clarkson & Greenwell* (1958), and minor endemics of erythromycin resistant *S aureus* (*Wickman* 1958) and neomycin resistant *S aureus* (*Lowbury et al* 1964) have been reported

Decoulx et al. (1965) found that on the 15th day after admission to a burns unit 78 per cent of the patients harboured in their burns *S aureus* of a phage type which had been demonstrated at admission in only 7 per cent

In a series of patients with burns treated during the period 1964–65, *MacMillan* (*1967*) found that 94 per cent harboured *S aureus* which in 83 per cent was of a phage type characteristic of the unit

Shallard & *O'Connor* (1967) studied *S aureus* isolated in a children's burns ward Out of 419 strains 135 were resistant to penicillin, streptomycin, erythromycin, kanamycin, and neomycin Most were non typable by the bacteriophages used

Below, the distribution of the *S aureus* isolated in the Burns Unit, Copenhagen, by phage type as well as by resistance will be discussed

MATERIAL

The patient material and therapeutic principles in the Unit have previously been described (*Thomsen* 1970 a and *Sorensen* & *Thomsen* 1968) With a view to epidemiological studies, the patients may be divided into 1676 not previously hospitalized patients and 305 patients who had previously been in contact with hospital environment having been transferred from another hospital or previously been admitted to the Burns Unit (*Thomsen* 1971) The Unit opened in November 1961, and the material comprises all patients admitted from that time until the end of 1968

METHODS

The phage typing was carried out in the State Serum Institute, Department of Diagnostic Bacteriology, by methods described by *Rosendal* (1959), *Rosendal et al* (1963), and *Rosendal* & *Bulow* (1967) All the strains of *S aureus* isolated from burns in the Unit were phage typed In addition a number of strains isolated in 1962 and 1963 were re typed in 1969 In classifying the phage types the following grouping was used

The complex 52, 52A, 80, 81 comprises the combinations 52/52A/80/81 52/52A/80 80/81 as well as types 80 and 81 Other types and combinations belonging to group I are listed under "rest of group I"

Type 83A/84/85/6557 comprises a few strains which were designated as 83A or 83A/6557 during the first years, as all subsequently re typed strains have proved to be lysed also by 84, 85 and 6557 (*Bulow* 1969 a)

The complex 84, 85, 6557 comprise strains which are lysed by this combination as well as types 84/85 and 6557 *In addition, it comprises all the previously re typed NT strains which were lysed by 6557* and among which the subsequently re typed ones have proved to be lysed also by 84 and 85 and in most cases also by 592 (*Jessen et al* 1969)

The designation "rest of group III" covers all other combinations of phage types from this group

NI (non identified) refers to strains that could not be referred to a single serological group Miscellaneous signifies phage types which cannot be assigned to any of the groups, in the present material predominantly type 187

NT means strains which could not be lysed by either standard set of phages or by phage 6557

The sensitivity to antibiotics was determined by the methods previously described by the present author (*Thomsen* 1971)

In the determination of the sensitivity to various

TABLE 1 *The Burns Unit in Copenhagen 1961 68 Occurrence of Various Phage Types of* S aureus *Isolated from the Nose and Throat at Admission*

	Isolated from the nose in percentage of %		Isolated from the throat in percentage of %		No of pts with the same phage type in the nose or throat and burns		Percentage clinically infected	
	Prim adm	prev hosp	Prim adm	prev hosp	Prim adm	prev hosp	Prim adm	prev hosp
52 52A 80 81	8	11	9	4	7	5	5	4
rest of gr I	15	10	13	12	9	3	4	3
group II	20	24	26	19	15	4	8	4
83A/84/85/6557	2	10	3	10	0	10	1	6
84, 85 6557	3	14	1	12	3	8	1	6
rest of gr III	15	14	21	23	16	7	10	3
NI + misc	11	6	12	8	12	2	1	1
NT	26	16	14	8	6	6	4	5
No of pts	473	88	209	43	68	15	41	32
Not phage typed	16	9	7	3				
Percentage of all admitted cases					4%	15%		

antibiotics described in the last section, the tube dilution method with two-fold dilution series in 2 ml liquid broth was used Unless otherwise stated, the inoculum was 0,1 ml of an overnight broth culture diluted 1 100 (approx 10^6 bacteria) Reading of the minimal inhibitory concentration (MIC) was *done* grossly after 24 and 48 hours incubation at 37° C and confirmed by sub cultures on broth agar plates

RESULTS

Phage Types of Staphylococcus aureus *Isolated in the Burns Unit*

As reported previously (*Thomsen* 1970 a), swabs were taken at admission from the nose, throat, and burns in all cases This revealed *Staphylococcus aureus* in the noses of 489 out of the 1676 patients who had not previously been hospitalized, in 216 in the throat, and in 98 in both sites Among the 305 patients transferred from other hospitals or previously admitted to the Burns Unit, *S aureus* was demonstrated at admission in 97 in the nose, in 51 in the throat, and in both sites in 35 The distribution of the phage types is listed in Table 1 Phage typing is available for nearly all strains, and in patients who had not previously been hospitalized this distribution must express the normal occurrence of various phage types among nasal staphylococci in a population in which 39 per cent were under 5 years of age, 12 per cent between 5–15 years 38 per cent from 16–59 years and 11 per cent over 60 (*Thomsen 1970* a) Only 3 per cent of the patients had *S aureus in* the burns already at admission usually of the same phage type as in the nose or throat Table 1 gives the number of patients whose burns at some time or other, harboured *S aureus* of the same phage type as that present in the nose or throat at admission (3rd column) It also shows how many of these patients developed clinical infection (4th column)

In a previous paper (*Thomsen* 1971) the antibiotic sensitivity of *S aureus* isolated in the Burns Unit was described Strains isolated from the noses of patients not previously hospitalized were found to be resistant to penicillin in only 57 per cent of the patients who were nasal carriers at admission Only a few per cent harboured multiresistant *S aureus* at admission This fits in with the phage types demonstrated in *S aureus* from the nose and throat Only strains belonging to the complex 52, 52A, 80, 81 were frequently *resistant to* penicillin as well as to streptomycin The common occurrence of type 83A and the *complex* 84, 85, 6557 in *S aureus* from previously hospitalized patients corresponds to the fact that multiresistant *S aureus* strains were isolated in one third of these patients at admission These phage types were also commonly found in *S aureus* strains from the nose and throat as well as from the burns of previously hospitalized patients and were in most cases accompanied by clinical infection

During the stay in hospital no routine cultures were obtained from the patients noses, and only at long intervals from the staff In a number of cases, however, the in patients showed *S aureus* of another phage type than at admission, during the first year especially 52/52A/80/81, but more recently 84/85/6557 Among the staff, *S aureus* of phage type 52/52A/80/81 was found in all cultures obtained in the period from 1962 to 1966 from the nose of a nurse who had been employed in the Unit from the time when it opened in November 1961 On each occasion, this strain was *resistant to penicillin* and streptomycin Apart from this, repeated cultures have not shown *S aureus*, neither in the staff nor on various utensils

Table 2 lists the annual occurrence of phage types of *S aureus* isolated from *patients in* the Burns Unit Only during the first year (from November 21st and throughout 1962) did the complex 52, 52A, 80, 81 predominate in the *burns, whereas the complex 84, 85,* 6557 has been constantly on the increase Type 83A was seldom demonstrated

In Table 3 these phage type patterns are related to the sensitivity of the isolated strains of *S aureus to* antibiotics Most strains belonging to the complex 52, 52A, 80, 81 have been resistant to penicillin *and streptomycin* (PS) *S aureus* of phage type 83A was most

TABLE 2 *The Burns Unit in Copenhagen 1961 68 Occurrence of Various Phage Types of* S aureus *Isolated from Burns*

	1961 62	1963	1964	1965	1966	1967	1968	Mean
	Demonstrated in percentage of patients harbouring *S aureus* in the burns							
	%	%	%	%	%	%	%	%
52, 52A 80, 81	35	6	11	14	8	5	1	11
rest of gr I	4	11	16	12	6	9	5	9
group II	16	8	7	10	16	3	11	10
83A/84/85/6557	9	7	2	9	6	5	2	6
84, 85, 6557	20	58	36	51	45	80	72	53
rest of gr III	21	18	34	13	19	6	10	16
NI + misc	9	14	9	7	10	5	10	9
NT	7	16	11	8	13	5	4	9
No of pts with *S aureus*	87	95	56	77	62	80	107	564

TABLE 3 *The Burns Unit in Copenhagen 1961 68 Sensitivity to Antibiotics in* S aureus *of Various Phage Types Isolated from Patients with Burns*

	No of pts	Percentage of patients with *S aureus* resistant to				
		sensit %	P %	PS %	PST %	PSTE*) %
52 52A 80 81	64	22	9	61	5	2
rest of gr I	42	62	43	5	0	5
group II	56	23	75	0	2	0
83A/84/85/6557	32	3	19	0	47	28
84, 85 6557	302	2	2	<1	5	93
rest of gr III	83	17	60	1	5	12
NI + misc	42	40	50	0	2	7
NT	52	31	29	6	21	13

*) P = Penicillin S = Streptomycin
T Tetracyclines E = Erythromycin

often resistant to penicillin, streptomycin, and tetracyclines (PST), while nearly all strains lysed by the new bacteriophages 84, 85, and 6557 were also resistant to erythromycin (PSTE)

Initially 83 patients harboured in their burns *S aureus* of one phage type which later was replaced by others presumably indicating cross infection The number of these patients exceeds that of patients in whom a strain with altered sensitivity to antibiotics was demonstrated The fact is that a number of the secondarily isolated strains showed the same sensitivity as those demonstrated first, in spite of an altered phage type Table 4 shows, to the left, the number of patients in whom *S aureus* of different phage types was demonstrated primarily To the right, the Table lists patients in whom *S aureus* of altered phage type was demonstrated. In most patients the primarily isolated strain had been replaced by one of the 84, 85, 6557 complex owing to the endemic presence of these types in the Burns Unit Only 6 patients had primarily *S aureus* of the 84, 85, 6557

TABLE 4 *The Burns Unit in Copenhagen 1961 68 Number of Patients Whose Burns Harboured* S aureus *of More Than one Phage Type Distributed According to Time of Isolation*

	Isolated primarily	Isolated secondarily in
52 52A, 80 81	12	6
rest of gr I	15	0
group II	13	4
83A/84/85/6557	11	5
84, 85 6557	6	69
rest of gr III	16	8
NI + misc.	7	2
NT	8	0

complex, which later was replaced by another strain, in most cases of the type 83A/84/85/6557 However, *S aureus* of several phage types were often present at the same time in the burn

To elucidate differences in the pathogenicity of the different phage types of *S aureus*, I collected those patients who showed a good take when the burns were grafted (90 100 per cent of the applied skin grafts) The phage types of *S aureus* strains isolated from these burns were compared with the corresponding ones in patients in whom the result of transplantation was so poor that the takes were assessed as less than 75 per cent Complex 52 52A, 80, 81 as well as type 83A and complex 84, 85, 6557 were more common in *S aureus* from patients with poor takes

Phage Types in Relation to Antibiotic Sensitivity

When the demonstrated phage types of the *S aureus* strains isolated in the Burns Unit are correlated with the sensitivity to antibiotics, it is apparent that already a short time after the Unit opened the predominant strain belonged to the phage type complex 84 85 6557 and was resistant to penicillin, streptomycin, tetracyclines, and erythromycin (PSTE) As previously reported, all *S aureus* strains isolated during the period 1961–1965 were stored in agar stab cultures, and in 1968 they were again tested for sensitivity to a number of antibiotics (*Thomsen* 1971) In addition those strains which previously could not be lysed by the bacteriophages used were re typed if they had been resistant to erythromycin

This revealed that strains which previously had been lysed only by phage 83A were now also lysed by the bacteriophages 84, 85 6557 and 592 This also applied to the strains isolated during recent years in the Burns Unit and which primarily could be typed as 83A/84/85/6557

As is apparent from Table 2 phage type 83A has been demonstrable in *S aureus* isolated from a few per cent of the patients harbouring *S aureus* in their burns but with decreasing frequency in the course of time The resistance pattern has varied (Table 3), and *S aureus* of this phage type has been resistant to erythromycin in only 28 per cent of the patients (*Bulow* 1968 b) Practically all have been resistant to PST and fully sensitive to other antibiotics, in particular to neomycin and bacitracin (*Rosendal* & *Jessen* 1964) In addition, the intervals between the demonstrations have been too long for the individual findings to be explicable as a result of cross infection in the Burns Unit

S aureus of phage type 84/85/6557/592 (originally designated as non typable) was demonstrated for the first time in the Burns Unit on January 15th, 1962, it spread rapidly and predominated the sensitivity patterns

All multiresistant (PST) strains of *S aureus* isolated in the Burns Unit from January 1962 to the end of 1965 were tested in 1968 for sensitivity to a number of antibiotics (*Thomsen* 1971) Out of a total of 472 strains 261 proved resistant to erythromycin Nine of these strains were of phage type 83A, the remainder were primarily designated as NT, but when tested with the new bacteriophages they were lysed either by 6557, which was first used alone, or when tested later also by 84, 85, and 592 A few strains were lysed at the primary test at the time of their isolation only by phages in undiluted suspension but have later been re typed as 84/85/6557/592 or 6557 All strains belonging to the complex 84 85, 6557 were resistant to neomycin and bacitracin and this also applied to the few strains isolated as early as January 1962 Only two strains isolated in June 1963, were resistant to neomycin but sensitive to bacitracin On the other hand the 9 strains which were lysed by 83A were sensitive to neomycin as well as to bacitracin

Those strains of *S aureus* which on sensitivity determination by the paper disc method proved resistant to erythromycin were studied throughout 1962 and during part of 1963 by the tube dilution method The minimal inhibitory concentration (MIC) of these 76 erythromycin resistant strains proved to

be in the range from 250 to more than 1000 μg/ml In half the cases growth was demonstrated at these high concentrations only after 48 hours incubation Erythromycin sensitive strains isolated during the same period were totally inhibited by *concentrations of* 0,5 1 μg/ml

The same 76 strains isolated in 1962 63 were also studied for sensitivity to neomycin and bacitracin by the tube dilution method

Using neomycin the MIC of sensitive strains was 6 25 μg/ml while resistant strains were not inhibited until at concentrations exceeding 1000 μg/ml In several cases total inhibition of growth (bactericidal effert) was not obtainable at all (cf the fact that the solubility of neomycin in water is about 6 mg/ml)

Using bacitracin the MIC of sensitive strains ranged from 25 to 100 μg/ml, while the resistant strains were inhibited by concentrations of 400 μg/ml and over Most strains showed inhibition of growth at 800 μg/ml, but a bactericidal effect did not occur until at concentrations of 1000 2000 μg/ml

Thus, using all 3 antibiotics there was a distinct difference between sensitive and resistant strains, and no gradual transitional forms between sensitive and resistant strains could be demonstrated in these early strains, neither to neomycin nor to bacitracin

A selection of erythromycin sensitive as well as erythromycin resistant strains proved upon determination of sensitivity to spiramycin to have a MIC of 16 50 μg/ml

Using oleandomycin erythromycin sensitive strains showed a MIC of 4 μg/ml while a selection of erythromycin resistant strains had a MIC of 15 60 μg/ml but not until after incubation for 48 hours In the sensitivity tests using paper disc and incubation for 24 hours these strains had been evaluated as *sensitive to oleandomycin* but showed a zone of inhibition which was smaller than that in fully sensitive strains During the latter half of 1968 *S aureus* of phage type 84/85/6557—totally resistant to penicillin, streptomycin, tetracyclines erythromycin neomycin bacitracin as well as to lincomycin, oleandomycin and spiramycin—were isolated in the Burns Unit These strains occurred in the Unit simultaneously with strains of the same phage type which were resistant to PSTENB but sensitive to lincomycin oleandomycin and spiramycin During the years 1966 and 1967 *S aureus* was not studied for sensitivity to neomycin, bacitracin lincomycin, oleandomycin or spiramycin

DISCUSSION

As already mentioned in the report on the antibiotic sensitivity of *S aureus* isolated in the Burns Unit, Copenhagen auto infection seldom occurred in the burns Most of the strains which in the case of nasal carriers infected the burns were of the complex 52 52A, 80 81 According to *Haynes et al* (1960), most *S aureus* isolated from burns belonged to phage types of group III They seldom found the type in the burns to be the same as that the staff carried in their noses and throats The cultures of samples obtained from the staff in the Burns Unit, Copenhagen, are too few to permit a conclusion However it is striking that only one nurse was found to be a nasal carrier of *S aureus* This strain was type 52/52A/80/81 and persisted as mentioned above for a number of years while, on the whole this type was decreasing in the Unit

The change in the predominant phage type pattern demonstrated in *the Burns Unit* corresponds exactly to the findings obtained by *Rosendal & Bulow* (1967) in the whole of Denmark *MacMillan* (1967) also found that *S aureus* of type 80/81 in a burns unit gradually was replaced by strains lysed by the experimental phage UC18 In part these strains became more widespread in the unit and in part they gradually superseded strains of other types in the patients burns

It is difficult to state whether the endemic spread of type 84/85/6557 with the resistance pattern PSTENB is the result of a selection owing to the extensive use of neomycin and bacitracin for local treatment or whether it is a consequence of actual emergence of resi

stance due to the use of these antibiotics According to *Jevons et al* (1966), the resistance to neomycin and bacitracin had contributed to the rapid spread of these strains in the hospital environment Since erythromycin has never been used in the Burns Unit, resistance to this agent can at least be explained only by the presence of the endemic type

Bulow (1968 c) has pointed out that genetic markers for resistance to various antibiotics may be coupled by genetic linkage and that a number of such coupled properties as regards resistance, may afford the multiresistant strains certain advantages above others resistant to fewer antibiotics

Bulow (1968 b) also demonstrated in a Copenhagen hospital, epidemic spread of type 6557 during 1962 At the same time type 83A/6557 decreased in frequency *Bulow* (1968 a) interpreted this as suppression of 83A by 6557 due to lysogenic conversion This blocking of the sensitivity to phage 83A was accompanied by resistance to erythromycin, neomycin and bacitracin Neomycin bacitracin resistance was found to be combined only with the inducible form of resistance to erythromycin

In the present material from the Burns Unit the erythromycin resistance was also of the dissociated form in accordance with the fact that erythromycin has never been used in the Burns Unit The resistance to this agent must therefore be considered a result of the widespread use of neomycin bacitracin which has contributed to the great spread of the endemic type in the Unit

Up to the end of 1965 all erythromycin resistant *S aureus* strains in the Burns Unit were sensitive to oleandomycin although the rather high MIC values indicate incipient selection Distinct cross resistance was not demonstrated until 1968 However *S aureus* strains from the intervening period have not been preserved and thus retrospective studies are out of the question

As demonstrated by *Garrod* (1957) the occurrence of cross resistance between oleandomycin and erythromycin is extremely varying in clinical materials, but often the same in the same hospital *English & Fink* (1961) found the MIC of oleandomycin in sensitive *S aureus* to be 3 6 μg/ml However, a few strains were not inhibited until at 12 50 μg/ml, and only a few were resistant to concentrations exceeding 100 μg/ml In *Jansson & Wager's* (1962) material 7 per cent of the studied *S aureus* strains were highly resistant to oleandomycin

Kefalides et al (1964) found in *S aureus*, isolated from burns in children, a total cross resistance between erythromycin and oleandomycin, although this latter antibiotic had never been used in the treatment of patients with burns *Lowbury & Hurst* (1959) could not demonstrate cross resistance between erythromycin and oleandomycin in *S aureus* isolated from burns

CONCLUSION

S aureus of the complex 84, 85, 6557 has been demonstrated with increasing frequency, partly in the Burns Unit, Copenhagen, as a whole and partly in the individual patients Its frequency increased with increasing duration of infection, it predominated over other types of *S aureus* which frequently were replaced by this strain, and occurred more often in patients with poor takes of skin grafts

On the basis of the present material it is difficult to assess the virulence in *S aureus* of phage type 84/85/6557 The great tendency to spread, however, is clearly apparent but as demonstrated by *Williams* (1963) this need not bear relation to its possible virulent properties The high frequency of 84, 85, 6557 in clinically infected patients may be due to these patients' longer stay in hospital and thus shows merely the contagious properties of these types

The author is indebted to *Kirsten Rosendal* M D for the permission to use the results of the phage typing and for guidance in the classification

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IN VITRO ACTIVITY OF GENTAMICIN, ALONE AND IN COMBINATION WITH OTHER ANTIBIOTICS

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A number of *in vitro* investigations have been carried out with gentamicin Kinetic experiments were performed on the antibacterial effect of gentamicin alone and in combination with seven other antibiotics Chloramphenicol and tetracycline inhibited the bactericidal effect of gentamicin whereas the combination of gentamicin with three penicillins sulphathiazole polymyxin B and streptomycin showed an additive effect by inhibiting regrowth of the test organism The antibacterial spectrum of gentamicin was examined, based on IC50 determinations of 158 gram positive and 266 gram negative strains In addition 519 *Staphylococcus aureus* strains were tested 516 of which were regarded as being fully sensitive Comparison was made of the relationship between the sensitivity of gentamicin and streptomycin kanamycin, and neomycin A high degree of concordance between gentamicin and neomycin was found although this was not complete Streptomycin and kanamycin showed less correlation with gentamicin In three strains which had been made resistant to gentamicin a similar reduction in sensitivity to the other antibiotics developed A method is described for routine sensitivity testing, using prediffusion and discs containing 25 µg gentamicin sulphate

Gentamicin is a relatively new antibiotic from actinomyces *Micromonospora purpurea* described by *Weinstein et al* in 1963 (24)

Chemically gentamicin belongs in the group of aminoglycoside antibiotics which also comprises streptomycin neomycin kanamycin and paromomycin Its structural resemblance to the other aminoglycosides seems to include a corresponding antibacterial mechanism of action which involves misreading of messenger RNA template of protein synthesis (6 10)

In vitro gentamicin has a bactericidal effect in concentrations obtainable in serum and a broad antibacterial spectrum Of particular interest is the activity *Pseudomonas aeruginosa* (18) and *Staphylococcus aureus*, especially the multiresistant strains isolated in hospitals (2)

Clinically it has been demonstrated that gentamicin is effective in the treatment of infections of the urinary system caused by numerous gram negative bacteria, including *P aeruginosa* (5) Reports have also been published regarding its effect on other forms of infection such as septicaemia (13) meningitis (15), and pulmonary infections (3)

Severe vestibular damage has been observed after treatment with gentamicin (11) The otological side effects seem to occur only when the concentration of gentamicin in

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serum exceeds 12 μg/ml The majority of the cases of vestibular side effects observed were reversible The question whether gentamicin causes significant nephrotoxic side effects is still open (8 14)

The present paper reports the results of *in vitro* experiments with gentamicin, including examination of its antibacterial spectrum, development of resistance, occurrence of cross resistance, and its effect in combination with other antibiotics An account is also given of a method for routine sensitivity determination to gentamicin

MATERIAL AND METHODS

Strain

The material consists of freshly isolated strains from specimens sent in for culture and sensitivity test to the Department of Antibiotics, Statens Seruminstitut, during 1967 68, and also some lyophilized laboratory strains

Antibiotics

Gentamicin sulphate (587 μg base/mg sulphate), Schering
Streptomycin sulphate (725 μg base/mg sulphate) Leo
Kanamycin sulphate (794 μg base/mg sulphate), Lundbeck
Polymyxin B sulphate (670 μg base/mg sulphate) Novo
Neomycin sulphate (700 μg base/mg sulphate) *Lundbeck*

(All weights of these drugs are given as sulphate in this report if not other ...

... penicillin and carbenicillin were dissolved in aqueous phosphate buffer, pH 6 5 and the other drugs in sterile water

The gentamicin solutions were stored at 4°C for a maximum of 5 days while the other solutions were used on the day of preparation

Media

5 per cent blood agar was used in the plate dilution method and 10 per cent blood agar without peptone in the diffusion experiments (21)

Seitz filtered meat broth containing 0 3 per cent sodium chloride, 0 2 per cent secondary sodium phosphate (Na_2HPO_4 $12H_2O$) and 1 per cent peptone was used in the resistance development and combined effect experiments Meat broth plates (1 8 per cent Japanese agar) were employed for bacterial counting The pH of the media was 7 3–7 4

The strains were subcultured on meat broth agar

Discs

Paper discs, diameter 6 mm (Schleicher & Schuell, No 2668) were imbibed with the drug solutions dried at 40°C, and stored at −22°C until use (21)

50 per cent Inhibitory Concentration (IC50)

Determination of the IC50 for the individual strains was carried out by the plate dilution method (17) and the calculations were made according to the Karber method (9)

Development of Resistance

Development of resistance *in vitro* was achieved by subculturing the strains in broth containing increasing concentrations of gentamicin From the tube with the highest content of drug and full growth, a loopful of suspension was subcultured for a new dilution series containing increasing concentrations of gentamicin

Cross Resistance

The prediffusion method (21) was used for determination of cross resistance to other antibiotics The discs contained 50 μg per disc of streptomycin, kanamycin and neomycin and 10 μg per disc of gentamicin

Kinetic Study of the Effect of Gentamicin Alone and in Combination with Other Antibiotics

A laboratory strain *Esch coli* NCIB 8879 was used 100 ml Erlenmeyer flasks containing 50 ml of meat broth were placed in water bath at 37°C until the broth had reached that temperature A bacterial suspension in sterile 0 9 per cent saline containing about 10^9 bacteria per ml was prepared from an 18 hour-old plate culture This was diluted with sterile saline as required and the control and experimental flasks were inoculated with 0 5 ml of suspension 0 5 ml of the antibiotic solutions was added to the experimental flasks at different times after inoculation In the experiments with combinations of antibiotics these were added at the same time Oxidation and stirring were carried out in connection with the addition of drugs and taking of samples

Before the drugs were added a sample was taken for viable count and further counts were made 1 2 3 4 5 and 24 hours after addition of the antibiotics Ten fold dilutions were made with

hysiological saline and two plates were each inoculated with 0 1 ml of suitable dilutions The plates were incubated at 35°C for 16 18 hours after which counting was performed on plates containing from 60 to 600 colonies In the case of the low bacterial counts the colonies were counted from 0 1 ml of the undiluted medium, though this figure might be less than 60 The antibiotic present was eliminated by means of diluting

Determination of Regression Curves

Inhibition zones were determined for 153 strains by the prediffusion method IC50 values for 144 strains were within the range 0 05 and 100 µg gentamicin per ml Nine strains had IC50 values less than 0 05 µg/ml Discs containing 100, 50 25 and 10 µg gentamicin were used for the experiment

RESULTS

The IC50 values for 266 gram negative and 158 gram positive bacteria are shown in Tables 1 and 2 together with the variations between the strains and the median values

The least sensitive of the species examined were in the following order *Streptococcus faecalis*, *Streptococcus pneumoniae*, non haemolytic streptococci *Streptococcus pyogenes*, *Providencia* The most sensitive were *Staphylococcus albus* *Staphylococcus aureus*, *Listeria monocytogenes*, *Klebsiella ozoenae* *S albus* was somewhat more sensitive than *S aureus* In addition to the 70 strains of *S aureus* (Table 1), 519 strains of *S aureus* isolated consecutively in 1968 were tested by the prediffusion method Among these strains, 516 showed zone size corresponding to an IC50 value less than 0 5 µg gentamicin base per ml, and only 3 strains showed slightly reduced zone size corresponding to IC50 value between 0 5 and 5 µg gentamicin base per ml

71 per cent of the 58 *E coli* strains examined were inhibited by concentrations between 0 4 and 0 8 µg/ml 138 strains of *P aeruginosa* selected at random from strains isolated in the routine laboratory were investigated and showed IC50 values from 0 4 to 12 5 µg/ml The distribution of IC50 values was unimodal, the median being 2 4 µg/ml Less than 5 per cent had IC50 smaller than 0 8 µg/ml, while approximately 90 per cent of the strains were inhibited by 8 µg gentamicin sulphate per ml

Comparison of the activity of streptomycin, kanamycin, and neomycin on the one hand, and gentamicin on the other, on 149 different strains was made by means of the sizes of the inhibition zones obtained by the prediffusion method Fig 1 shows the correlation between the zone sizes obtained by streptomycin and gentamicin Strains without zones to streptomycin showed zones of varying sizes to gentamicin, whereas all strains without zones to gentamicin showed reduced zones to streptomycin also Fig 2 shows the same as regards kanamycin and gentamicin, where good correlation was found Three strains with relatively large zones to kanamycin showed no zones at all to gentamicin, while five strains without zones to kanamycin showed quite large zones to gentamicin Fig

TABLE 1 *Sensitivity of Gram Positive Bacteria of Gentamicin*

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		Range limits	Median value
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S albus	28	<0 1 0 4	0 05
S pneumoniae	10	12 5–25 0	17 5
S pyogenes	16	0 1 50 0	2.2
S faecalis	20	0 2 50 0	17 5
S non haemolyticus	4	0 4– 6 2	3 7
C d phtheriae	2	<0 1 0 2	
L monocytogenes	8	<0 1 0 2	

serum exceeds 12 μg/ml The majority of the cases of vestibular side effects observed were reversible The question whether gentamicin causes significant nephrotoxic side effects is still open (8, 14)

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Neomycin sulphate (700 μg base/mg sulphate) Lundbeck
(All weights of these drugs are given as sulphate in this report if not otherwise stated)
Tetracycline hydrochloride DAK Laboratory
Chloramphenicol, DAK Laboratory
Benzylpenicillin sodium Leo
Disodium-carbenicillin, Astra
Sulphathiazole sodium

G penicillin and carbenicillin were dissolved in aqueous phosphate buffer, pH 6 5 and the other drugs in sterile water

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74 per cent of the 58 *E. coli* strains examined were inhibited by concentrations between 0.4 and 0.8 µg/ml. 138 strains of *P. aeruginosa* selected at random from strains isolated in the routine laboratory were investigated and showed IC50 values from 0.4 to 12.5 µg/ml. The distribution of IC50 values was unimodal, the median being 2.4 µg/ml. Less than 5 per cent had IC50 smaller than 0.8 µg/ml, while approximately 90 per cent of the strains were inhibited by 8 µg gentamicin sulphate per ml.

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TABLE 1. *Sensitivity of Gram Positive Bacteria of Gentamicin*

Strains	Number of strains	IC50 (µg/ml)	
		Range limits	Median value
S. aureus	70	0.1– 0.8	0.2
S. albus	28	<0.1 0.4	0.05
S. pneumoniae	10	12.5–25.0	17.5
S. pyogenes	16	0.1 50.0	2.2
S. faecalis	20	0.2 50.0	17.5
S. non haemolyticus	4	0.4 6.2	3.7
C. diphtheriae	2	<0.1 0.2	
L. monocytogenes	8	<0.1– 0.2	

TABLE 2 *Sensitivity of Gram Negative Bacteria to Gentamicin*

Strains	Number of strains	IC50 (µg/ml)	
		Range limits	Median value
E coli	58	0 2– 3 2	0 8
Klebsiella	59	<0 1– 1 6	0 5
Enterobacter	20	0 2– 1 6	0 6
Serratia	19	0 2 0 8	0 6
Proteus	38	0 4– 3 2	0 9
Providencia	6	3 2–50 0	8 8
Citrobacter	16	0 2 0 8	0 6
P aeruginosa	138	0 4 12 5	2 4
Pseudomonas spp	4	0 1–>50	
Aeromonas	3	0 4 0 8	

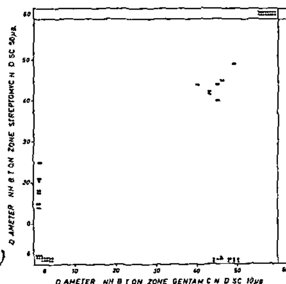


Fig 1 Correlation between diameter in mm of inhibiting zones of 149 strains using 50 µg neomycin discs and 10 µg gentamicin discs and 20 hours prediffusion

3 shows the correlation between neomycin and gentamicin where good correlation was obtained However there was not not complete parallelism between neomycin and gentamicin since in another experiment 19 strains isolated from blood cultures that were resistant to neomycin and kanamycin were all sensitive to gentamicin

Development of resistance *in vitro* was determined with three strains (*S aureus E coli* and *P aeruginosa*) The inhibitory concentrations increased rapidly during the first subcultures but later a larger number of subcultures was necessary between each step (Fig 4)

The gentamicin resistant variants showed a parallel reduction in sensitivity to streptomycin kanamycin, and neomycin while there was no change in the sensitivity to novobiocin and polymyxin B

The death curves for *E coli* NCIB 8879 exposed to gentamicin both alone and in combination with other antibiotics, were determined in a number of experiments The IC50 for the strains was 0 8 µg/ml genta

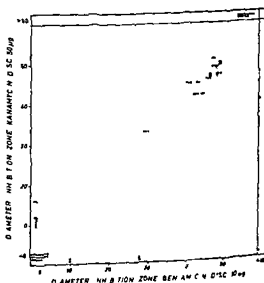


Fig 2 Correlation between diameter in mm of inhibition zones of 149 strains using 50 µg kanamycin discs and 10 µg gentamicin discs and 20 hours prediffusion

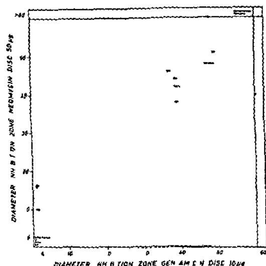


Fig 3 Correlation between diameter in mm of inhibition zones of 149 strains using 50 µg neomycin discs and 10 µg gentamicin discs and 20 hours prediffusion

micin The death curve was determined after addition of gentamicin at the commencement and at the end of the lag phase and both early and late in the logarithmic growth phase After the addition of gentamicin to give a final concentration of 2 µg/ml a death curve at that shown in Fig 5 was found in all experiments The bactericidal effect became apparent immediately after the addition and the number of bacteria was reduced 10^4 to 10 times during the first hours There was no subsequent further decrease but instead secondary growth occured which brought the number of bacteria up to the same level as that in the control culture within 24 hours If 1 µg/ml gentamicin was employd there was a lesser bactericidal effect which ceased earlier and was followed by secondary growth If the concentration of gentamicin was increased to 10 µg/ml the death curve was initially the same as that described above, but the number of bacteria fell to zero after 5 hours and there was no secondary growth within 24 hours

The secondary growth was subjected to further examination The sensitivity to gentamicin of the secondary culture was reduced somewhat, the IC50 being increased from 0 8 to about 2 µg/ml The variants grew more slowly than the mother strain The generation time was about 31 minutes whereas under the same conditions the primary culture had a generation time of about 20 minutes Examination of the content of gentamicin in the broth by the agar cup method showed that the concentration fell from 2 0 to about 1 2 µg/ml in the course of 24 hours Measurement of pH in the culture revealed that the maximum changes during the experiment were less than 0 2 pH units

Addition of gentamicin (2 µg/ml) to the bacterial culture in the lag phase with inocula varying from 10^3 to 10^7 bacteria per ml caused death curves with almost unaltered appearance This would indicate an only slightly pronounced inoculum effect on the strain in question under the experimental conditions described

The result of simultaneous addition of gentamicin to a final concentration of 2 µg/ml in combination with other antibiotics to the culture in the lag phase inoculated with about 10^5 bacteria per ml is shown in Fig 5 The concentrations of the other drugs are stated in the figure The combinations gentamicin/penicillin gentamicin/streptomycin gentamicin/polymyxin B and gentamicin/sulphathiazole showed an additive effect Simultaneous addition of tetracycline

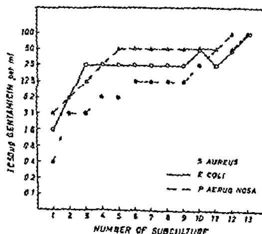


Fig 4 Development in vitro of resistance to gentamicin in three strains

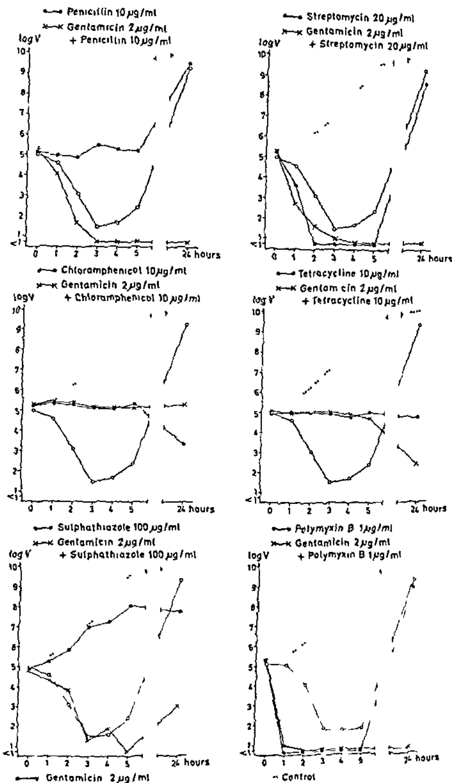


Fig 5 Death curves of *E coli* NCIB 8879 (inoculum 10^5 bacteria per ml) following exposure in the lag phase to gentamicin 2 µg/ml alone and in combination with six other antibiotics compared with a control without antibiotic *Concentrations of drug are stated in each section of the figure*

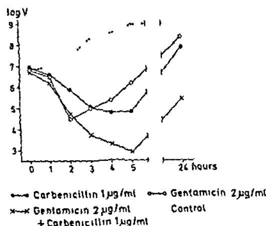


Fig 6 Death curves of *E coli* NCIB 8879 (inoculum 10^7 bacteria per ml) following exposure in the lag phase to gentamicin 2 µg/ml, carbenicillin 1 µg/ml and gentamicin 2 µg/ml + carbenicillin 1 µg/ml compared with a control without antibiotic

or chloramphenicol and gentamicin inhibited the bactericidal effect of gentamicin on *E coli*

Fig 6 shows the result of the combination gentamicin/carbenicillin i.e. an additive effect on *E coli* NCIB 8879

Fig 7 shows the correlation between the IC50 values and the inhibition zones in the prediffusion method (discs containing 25 µg gentamicin) Fully sensitive strains (+ + +) are those with IC50 less than or equal to 0 8 µg gentamicin sulphate per ml, corresponding to 0 5 µg gentamicin base per ml Moderately sensitive strains (+ +) are those with IC50 between 0 8 and 8 µg/ml (0 5 and 5 µg base) Relatively resistant strains (+) are those with IC50 between 8 and 50 µg/ml (5 and 30 µg base), and strains with IC50 greater than 50 µg/ml are considered resistant to gentamicin (0) The limits of sensitivity groups are chosen according to the concentrations usually obtained in serum After administration of 40 or 80 mg to an adult subject, the serum levels become 2 6 µg/ml (5, 13) In order to maintain a sufficient degree of security, only strains inhibited by less than one fifth of these concentrations are considered fully sensitive

DISCUSSION

The antibacterial spectrum of gentamicin as shown in Tables 1 and 2 is in agreement with previously published studies where the results with gentamicin base are given (1, 2, 23) An exception is *P aeruginosa* which showed IC50 values higher than the MIC values found by *Auwarter* & *Naumann* (1) using the tube dilution method As a small inoculum (200 500 bacteria) was used in the present investigation, lower IC50 values would be expected as compared to other experiments where a larger number of bacteria was employed However, the inoculum effect of gram negative bacteria is slight, whereas it seems to be pronounced for *S aureus* (1, 2) The divergent sensitivities of

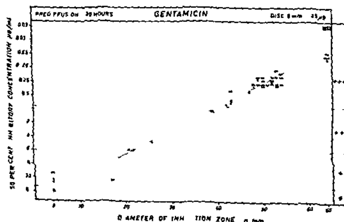


Fig 7 Regression curve for gentamicin between IC50 and diameter of inhibition zones obtained by the prediffusion method in 153 strains Horizontal lines indicate the limits between sensitivity groups

P aeruginosa strains might be due to the different media and oxidation conditions used in this study and in that of *Auwarter & Naumann*

Examination of a large number of *S aureus* strains isolated from clinical material in 1968 showed that these were fully sensitive to gentamicin and this applied also to strains resistant to kanamycin and neomycin *In vitro* development of resistance to gentamicin was found to be accompanied by simultaneous development of resistance to kanamycin (2) According to *Davies et al* (7), the development of resistance to gentamicin is determined by the fact that gentamincin is inactivated by phosphorylation by means of a periplasmic enzyme This enzymatic phosphorylation of gentamicin takes place less frequently than similar break down of the other aminoglycosides It is found among strains with resistance transfer factor

Comparison of the sensitivity of 149 strains to the aminoglycosides streptomycin kanamycin neomycin and gentamicin showed substantial concordance between neomycin and gentamicin Streptomycin and kanamycin showed less correlation with gentamicin

In the prediffusion experiments discs containing 10 μg gentamicin gave inhibition zones that were similar to those found with discs containing 50 μg of the three other aminoglycosides This is in agreement with the literature according to which the daily dosage of gentamicin normaly is 240 mg i e approximately one fourth of those of kanamycin and streptomycin However the activity must be seen in relation to the toxicity of the drugs Examination of the LD50 for mice showed that neomycin was the most toxic while gentamicin was three to six times as toxic as kanamycin depending on the mode of administration (16 24)

The kinetic examination of the effect of gentamicin on a strain of *E coli* was initially bactericidal usually with subsequent secondary growth which is in agreement with previous reports (18) If however the concentration of gentamicin was 10 μg/ml the regrowth was not observed within 24 hours From a clinical point of view this is important to acknowledge but on the other hand 10 μg/ml gentamicin is a concentration near to the toxic level which should not be exceeded in serum Furthermore the concentration used is more than ten times that of IC50 for the strain and such levels are usually difficult to maintain in serum

Experiments with gentamicin in combination with penicillin streptomycin sulphathiazole, and polymyxin B showed no particular change in the initial bactericidal effect but the secondary growth was prevented or inhibited to a large extent When the combination of gentamicin and carbenicillin was employed subsequent secondary growth was recorded although this was delayed compared to that seen when either of the two antibiotics was used alone The reason for the secondary growth observed in this study is presumably due to the small concentration of carbenicillin used since others using 50 μg carbenicillin per ml have stated that no regrowth occured when *P aeruginosa* was employed (20) Only few kinetic examinations with gentamicin in combination with other antibiotics have been carried out previously These showed a synergistic effect of gentamicin/colistin and gentamicin/carbenicillin against *P aeruginosa* (20 22), and a synergistic effect of gentamicin in combination with penicillin or ampicillin against *Proteus mirabilis* (4) Particularly the combination gentamicin/carbenicillin against *P aeruginosa* seems to provide important clinical implications (19)

Tube dilution experiments with combinations of antibiotics cannot be compared directly with the experimental method used in the present study since in the former method reading is made only after incubation for 16 24 hours If the bactericidal effect of combinations is examined by inoculation from the tubes the value of the tube dilution method is increased

Chloramphenicol and tetracycline prevented the bactericidal effect of gentamicin This is in agreement with studies on the effect of other bactericidal antibiotics in combination

with bacteriostatic drugs Tetracycline and chloramphenicol prevent the bactericidal effect of gentamicin by inhibiting the protein synthesis at an earlier stage than the attack of the aminoglycosides, in the case of chloramphenicol by preventing the elongation reaction of protein biosynthesis (10)

The correlation between the IC50 values and the diameter of the inhibition zones by the prediffusion method is sufficiently good to warrant the use of that method in routine determinations of sensitivity to gentamicin Discs containing 25 μg gentamicin were chosen, since their use ensures the largest spread in zone diameters within the range of IC50 values that can be regarded as clinically relevant The large zones obtained in very sensitive strains with this content of gentamicin might seem disadvantageous, but in practice this has proved to be of no significance Only strains with IC50 less than or equal to 0 5 base μg/ml (0 8 μg sulphate per ml) have been considered fully sensitive, in contrast to findings by *Kirby & Standiford* (12) who set the limit at 5 μg/ml The writers have found such a concentration too high, both from the point of view of the serum levels obtained after normal doses of gentamicin and the ototoxic side effects which might occur at concentrations higher than 12 μg gentamicin base per ml (11)

However, gentamicin might be used occasionally in the treatment of infections caused by strains with IC50 between 0 5 and 5 μg gentamicin base per ml In such cases, increased dosage is necessary and therefore determination of serum levels become obligatory In urine from patients with normal renal function the concentration of gentamicin is usually approximately 50 times that of serum This might justify use of the antibiotic, though the causative micro-organism has a rather high IC50

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Acta path microbiol scand Section B 79 343 344 1971

N-DEMETHYLSTREPTOMYCIN

3 *Antibacterial Activity*

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In a previous paper (1) it was stated that the microbiological activities of N demethylstreptomycin (NDMS) and dihydro N demethyl streptomycin (DH NDMS) were equal to about 10 per cent that of streptomycin These activities were estimated by the agar diffusion method using *Bacillus subtilis* ATCC 6633 as the test organism and were performed after each step during the recovery and purification of the compounds DH NDMS was recrystallized further until estimations made from the NMR spectrum showed a 95 per cent DH NDMS content and a 5 per cent impurity consisting solely of dihydrostreptomycin (DHS) This prompted us to make further investigations both *in vitro* and *in vivo* as to how the microbiological activity of the purified DH NDMS compared to that of DHS

MATERIALS AND METHODS

Cultures The following cultures were used *Staphylococcus aureus* ATCC 6538P *Staphylococcus aureus* ATCC 13709 *Staphylococcus aureus* 3356 3364 (clinically isolated strains) *Escherichia coli* 01 04 *Klebsiella pneumoniae* K8 *Proteus vulgaris* 5209 16868 (Gram neg strains from State Serum Institute Copenhagen Denmark)

Microbiological activity In vitro activities were determined by conventional tube dilution assays in Mycin Assay Broth (Difco) to obtain the minimum inhibitory concentrations (MIC) of the compounds

In vivo activities were determined by mouse assays in which the animals were infected intraperitoneally either with a 100 LD_{50} dose of *S aureus* ATCC 13709 or *E coli* 01 or 04 contained in 0 5 ml of 5 per cent hog gastric mucin All infected animals were treated *subcutaneously with a single* dose administered 1 hour after infection All medications were administered in water at four different levels of the test antibiotics at twofold increments Each treatment group consisted of 10 mice The animals were observed for 6 days and the ED_{50} was determined by the method of Reed and Muench[3]

Chromatography and NMR spectra Chromatography of DH NDMS was performed as described by Heding[2] Nuclear resonance spectra were determined using a Varian A 60 instrument Solutions of the compounds in deuterium oxide were lyophilized and the spectra determined after the compounds had been redissolved in the same solvent The ratio of the integrals of the $N\ CH_3$ ($\delta = 2\ 78$ s) and the $CH\text{-}CH_3$ ($\delta = 1\ 19$ d) groups was 5 per cent

TABLE 1 In vitro *Sensitivity of Selected Test Organism to DH NDMS and DHS*

Test organism	Minimum inhibitory concentration in µg/ml DH NDMS	DHS
S aureus 6538P	3 1	0 4
S aureus 13709	3 1	0 4
S aureus 3356	>200	200
S aureus 3364	6 2	0 8
E coli 01	3 1	1 6
E coli 04	6 2	3 1
P vulgaris 5209	6 2	3,1
P vulgaris 16868	12 5	3 1
K pneumoniae K8	1 6	0 4

Received 16 ix 70

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whether some of the commonly occurring yeast like fungi which cause yeast mycosis in human subjects are sensitive to Bay b 5097

MATERIAL AND METHODS

Strains

The following strains were included in the experiments *Candida albicans* (10 strains) 1) A 1822 2) A 1844, 3) A 1910, 4) A 2047 I, 5) A 2047 II, 6) H 2100 7) H 2130, 8) PF 2191, 9) PF 2357, 10) PF 2358

Candida parapsilosis (6 strains) 11) A 103 12) H 1551, 13) PF 1744, 14) H 1881, 15) H 1989, 16) PF 2079

Candida tropicalis (5 strains) 17) A 1906, 18) PF 2114 19) PF 2132 20) PF 2197, 21) PF 2199

Candida guilliermondii (4 strains) 22) H 1006 23) H 1137, 24) A 1925, 25) PF 2133

Candida pseudotropicalis (2 strains) 26) A 712, 27) PF 896

Candida parapsilosis variant *intermedia* (1 strain) 28) PF 2336

Candida rugosa (1 strain) 29) A 1738

Torulopsis glabrata (5 strains) 30) A 1933 31) A 1985, 32) A 2004, 33) PF 2150, 34) PF 2230

Cryptococcus neoformans (4 strains) 35) CBS 132, 36) Conant s strain 37) A 1839, 38) A 2317

Strain CBS 132 was received from Centraal bureau voor Schimmelcultures Yeast Division Delft, and strain 36 was kindly supplied by Dr Conant All the other strains were isolated from specimens sent to the Mycology Department, Statens Seruminstitut

None of the strains included in this study had been in contact with Bay b 5097 previously

Methods

For details regarding the production of the plates the inoculation technique the performance of the experiments and the antimycotics amphotericin B and nystatin used reference should be made to the previous study (*Bodenhoff* 1968 1969) As for the performance of the antimycetic Bay b 5097, a 0 1 per cent solution was made in ethanol

Other Examinations

During the experimental period the effect of temperature on the keeping qualities of Bay b 5097 was examined in medium stored at various temperatures

1) A dilution series consisting of Sabouraud agar plates with Bay b 5097 in the concentrations mentioned above was inoculated immediately after production with the cultures concerned

2) A corresponding dilution series to that in 1) was stored in tightly closed metal boxes for 8 and 16 days at 4° C before inoculation was performed

3) A dilution series as mentioned under 1) was stored as in point 2), except that storage was at 37° C for 8 and 16 days before inoculation was performed

RESULTS

The results of examination of the antimycetic effect of Bay b 5097 as compared with that of amphotericin B and nystatin are shown in Table 1 The column in the table marked "Min I C" gives the lowest concentration of antimycotic (Min I C = minimum inhibition concentration) that evokes complete inhibition The next column shows the highest concentration of antimycotic (Max G C = maximum growth concentration) at which the strain concerned still shows definite growth

The experiments show that none of the strains had natural resistance to Bay b 5097, but that there was great variability in the sensitivity to the drug not only from candida species to candida species, but also from strain to strain within the same species

It was observed during the experiments that crystals of Bay b 5097 became precipitated corresponding to the inoculated area on the surface of the plates containing 50 µg/ml of Bay b 5097

Evident inactivation of Bay b 5097 was observed in Sabouraud agar pH 6 stored at 4° C for 2 weeks (Min I C value 2 µg/ml as against 1 µg/ml for *Cryptococcus neoformans*, and Min I C value 25 µg/ml as against 10 µg/ml for *Candida albicans*)

DISCUSSION AND CONCLUSIONS

In their work from Bayer AG s laboratory *Plempel et al* (1969) state that after oral administration of Bay b 5097 to a group consisting partly of volunteers and partly of patients with mycosis a serum concentration of up to 7 µg/ml could be obtained How-

TABLE 1. *A New Antimycotic from Bayer AG, Bay b 5097*

Strain	Bay b 5097 µg/ml		Amphotericin B µg/ml		Nystatin µg/ml	
	Min I C	Max G C	Min I C	Max G C	Min I.C	Max G C
Candida albicans						
1 A 1822	25	10	2	1	10	5
2 A 1844	1	0.2	1	0.2	10	5
3 A 1910	10	5	1	0.2	10	5
4 A 2047 I	10	5	1	0.2	10	5
5 A 2047 II	10	5	1	0.2	5	4
6 H 2100	10	5	2	1	10	5
7 H 2130	25	10	1	0.2	10	5
8 PF 2191	2	1	1	0.2	4	3
9 PF 2357	5	4	1	0.2	5	4
10 PF 2358	25	10	1	0.2	10	5
Candida parapsilosis						
11 A 103	1	0.2	2	1	10	5
12 H 1551	3	2	2	1	10	5
13 PF 1744	2	1	2	1	10	5
14 H 1881	4	3	2	1	10	5
15 H 1989	2	1	2	1	10	5
16 PF 2079	25	10	2	1	25	10
Candida tropicalis						
17 A 1906	25	10	2	1	10	5
18 PF 2114	25	10	2	1	25	10
19 PF 2132	25	10	2	1	10	5
20 PF 2197	25	10	2	1	25	10
21 PF 2199	25	10	2	1	25	10
Candida guilliermondii						
22 H 1006	25	10	1	0.2	25	10
23 H 1137	5	4	1	0.2	10	5
24 A 1925	5	4	1	0.2	10	5
25 PF 2133	3	2	2	2	25	10
Candida pseudotropicalis						
26 A 712	2	1	3	2	10	5
27 PF 896	2	1	3	2	10	5
Candida parapsilosis var intermedia						
28 PF 2336	10	5	3	2	10	5
Candida rugosa						
29 A 1738	1	0.2	1	0.2	10	5
Torulopsis glabrata						
30 A 1933	5	4	1	0.2	5	4
31 A 1985	10	5	1	0.2	5	4
32 A 2004	4	3	1	0.2	4	3
33 PF 2150	4	3	1	0.2	4	3
34 PF 2230	4	3	1	0.2	4	3

TABLE 1 *(cont.)*

Strain	Bay b 5097 µg/ml		Amphotericin B µg/ml		Nystatin µg/ml	
	Min I C	Max. G C	Min I C	Max G C	Min. I C	Max. G C
Cryptococcus neoformans						
35 CBS 132	1	0 2	0 2	C*	2	1
36 Conant s strain	1	0 2	0 2	C*	1	0 2
37 A 1839	0 2	C*	0 2	C*	3	2
38 A 2317	1	0 2	1	0 2	4	3

In vitro sensitivity of 38 strains of the species *Candida*, *Torulopsis*, and *Cryptococcus* to Bay b 5097 in comparison with nystatin and amphotericin B

* Control plate (Sabouraud s maltose agar containing no antimycotic)

ever, about half of that concentration was said to be a metabolite that was ineffective against fungi It must be assumed, therefore, that the microbiologically active serum concentration achieved cannot generally be much over 3 µg/ml of Bay b 5097

The majority of the strains of yeast like fungi used in the present study were not inhibited by 4 µg Bay b 5097/ml with the technique employed In particular many—though not all—strains of *Candida albicans* and all the five *Candida tropicalis* strains were so little sensitive that they must be regarded as resistant

The poor sensitivity to Bay b 5097 observed in the experiments referred to is not in accordance with the results of *Plempel et al* (1969) who found that 61 out of 72 strains of *Candida albicans* and 6 out of 7 strains of *Candida tropicalis* were sensitive in a concentration of 1 µg/ml

The divergency between *Plempel et al* s results and those found in the present study may be due to the different technique used

My thanks are due to Farbenfabriken Bayer AG who has supplied me with the antimycotic Bay b 5097

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ANTIBODY-COMBINING OLIGOSACCHARIDES FROM A CHICK ALLANTOIC GLYCOPEPTIDE SULPHATE ASSOCIATED WITH INFLUENZA VIRUS HAEMAGGLUTININ

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Oligosaccharides that combine with antibody to a chick allantoic glycopeptide sulphate have been produced both by dilute acid and alkali treatment of the glycopeptide Chemical and immunological assays of the oligosaccharides in conjunction with periodate oxidation studies and enzyme treatments have helped to elucidate the carbohydrate residues responsible for antibody-combining activity in the parent glycopeptide Peptide, sialic acid, 2 amino 2-deoxyglucose and 2 amino 2-deoxygalactose residues in the glycopeptide play no part in conferring activity, but *D* galactose and to a lesser extent fucose are sole components of the active site Two types of *D* galactose determinant residues are present, differing in their susceptibility to periodate oxidation

The haemagglutinin of influenza virus grown in the chick allantoic sac is inhibited by antibody to normal (uninfected) chick allantoic material (12, 3) The inhibition is blocked by such material and a haemagglutination inhibition blocking (HIB) test was developed (4) for titrating the antigen concerned The inhibition of haemagglutination is due to the presence of the normal chick antigen in the influenza virus haemagglutinin (13)

Received 8 ix.70

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The purified antigen is a sulphated glycopeptide which contains D-galactose, fucose, 2 amino 2-deoxy galactose, 2 amino 2 deoxy glucose and acetyl groups (5, 6 7) Subsequent structural studies showed (8, 9, 10) that the antigen consists of a peptide "backbone" in which the β hydroxy groups of most of the serine and threonine residues are linked to 2 acetamido-2 deoxy galactose residues that are substituted at *O* 3 and *O* 6 by oligosaccharides The latter are terminated mainly, or exclusively by fucose and D galactofuranose residues and possibly, in a few cases by *D* galactopyranose residues *D* galactofuranose residues are also present in the interior of carbohydrate chains Spectroscopic evidence indicated the presence of axial rather than equatorial sulphate residues

The antibody-combining activity of the antigen is destroyed by treatment with an enzyme preparation from a soil bacterium (14) The present paper reports further studies of the immunochemistry of the antigen as revealed by HIB tests of fragments obtained from the antigen by treatment with acid, alkali, enzymes and sodium metaperiodate

MATERIAL AND METHODS

Chick Allantoic Antigen

Host antigen was prepared by a modification of the method described previously (5) and using allantoic fluid from the production of influenza vaccine In outline, the method involved removal of most of the virus by high speed centrifugation followed by dialysis, concentration and precipitation with Rivanol (6,9 diamino-2 ethoxy-acridine lactate) 2 per cent (w/v) The precipitate was dissolved in 0 3 M sodium chloride and Rivanol was removed by extraction with n butanol and dialysis Further purification was achieved by sequential precipitation with ethanol, chromatography on DEAE cellulose and DEAE-Sephadex and gel filtration on Sephadex G 150 The material thus obtained was homogeneous by gel filtration analytical ultracentrifugation, and by paper and free boundary electrophoresis

Chemical and Immunological Assays

Galactose and fucose were determined simultaneously by using the cysteine sulphuric acid assay (2) Neutral and basic monosaccharides in sample hydrolysates (2N hydrochloric acid 0 5 ml, 5 hr, 100°) were separated by elution from Dowex 50 (H^+) resin with water and 2N hydrochloric acid respectively, and were converted into trimethyl silyl (TMS) derivatives (1, 15) Derivatives were analysed by using a Pye 104 gas chromatograph and a column packing of 3 per cent SE 30 on siliconized celite (100–200 mesh Pye Unicam Ltd) at 180°

HIB Test

In the HIB test the material to be tested competes with influenza virus for antibody to the allantoic antigen thus blocking the inhibition of haemagglutination The B/Lee virus strain grown in White Leghorn eggs and purified by means of adsorption to and elution from fowl red cells, was used Immune sera against the allantoic antigen were prepared in rabbits by intramuscular injection of purified antigen in Freund s complete adjuvant (Difco) Non specific serum inhibitors were removed by treating serum (1 vol) with 0 01 M aqueous potassium metaperiodate (2 vol) The mixture was kept at 37° for 18 hr and 5 per cent glucose (2 vol) was added at room teperature The sera were then absorbed with a 10 per cent suspension of fowl red cells at 4°

The HIB test was performed in Perspex plates at room temperature Four haemagglutinating units of virus were used The immune serum was titrated in the haemagglutination inhibition (HI) test according to the method of *Isaacs et al* (11) and was diluted with phosphate buffered saline (PBS) to contain four HI units The material to be tested was diluted with PBS in serial two-fold steps and 0 25 ml immune serum was added to each dilution After 30 min 0 25 ml of 5 per cent suspension of fowl red cells was added followed by 0 25 ml of the virus suspension The mixture was left for the cells to settle and the pattern was read The HIB titre was defined as the reciprocal of the dilution giving 50 per cent agglutination The end point was usually well defined A standard solution of allantoic antigen was titrated as an internal standard and appropiate controls were included to investigate non specific haemagglutination

Sequential Treatment of Antigen with Sodium Metaperiodate and Sodium Borohydride

In a typical experiment antigen (2 mg) was treated with 0 01 M sodium metaperiodate (4 ml) in the dark at 4° After 24 hr, the excess of periodate was destroyed with ethylene glycol and the solution was dialysed against 0 1 M sodium chloride followed by distilled water at 4° The non diffusible material was reduced with sodium borohydride (3 mg) added during 3 hr The pH of the solution was maintained at 7 7 5 by using carbon dioxide After 18 hr at 4° and pH 7, Dowex 50 (H^+) resin (3 g) was added and the solution was stirred for 1 hr and filtered The filtrate was dialysed and the 'oxidized and reduced antigen was recovered in 85 90 per cent yield by freeze drying prior to analysis for monosaccharides and amino acids liberated on hydrolysis (8) Aliquots of an aqueous sterile filtered solution of the "oxidized and reduced antigen (500 μg/ml) were tested in the HIB reaction

Treatment of Antigen with Enzymes

The enzymes obtained from a Gram negative coccobacillus, were purified as described previously (14) Aliquots (0 05 ml) of solutions of the antigen (40 μg/ml) and the oxidized and reduced antigen (500 μg/ml) were mixed with solutions (0 5 ml) of the crude enzyme mixture and of partially purified enzymes derived from it in citric acid phosphate buffer, pH 7 and the solutions were incubated at 30° for 48 hr and then heated at 56° for 10 mins The solutions and the appropriate

controls were diluted with 0 9 ml PBS pH 7, and the HIB titres were determined These studies were repeated with the incorporation of 0 05 ml of 10 mM solutions of each of the following inhibitors 2 acetamido 2-deoxy *D* glucose 2 acetamido 2 deoxy *D* galactose *L* fucose *D* fucose and *D* galactose The solutions were diluted with 0 85 ml PBS prior to determining the HIB titres

Hydrolysis of Antigen with Dilute Acid

Allantoic antigen (56 mg) was hydrolysed (0 04N hydrochloric acid 15 ml 30 min, 100°) under nitrogen in a sealed tube and the hydrolysate was eluted with water from Sephadex G 25 (fine grade 148 × 2 8 cm) Fractions (4 4 ml) were collected automatically and were scanned for carbohydrate (2) The contents of the tubes containing material of apparently large molecular weight were pooled, concentrated by freeze-drying and rehydrolysed (0 08N hydrochloric acid, 5 ml, 30 min, 100°) and fractionated as above Material of apparently large molecular weight remaining after the second hydrolysis was also recovered rehydrolysed (0 08N hydrochloric acid 5 ml, 30 min, 100°) and the hydrolysate was eluted from Sephadex G 25 (fine grade, 123 × 1 3 cm)

Treatment of Antigen with Dilute Alkali

Allantoic antigen (51 6 mg) was treated with 0 2N sodium hydroxide (50 ml) under nitrogen in a sealed tube in the dark and the absorbance of the solution at 241 *nm* was determined at intervals during 32 hours at 25° The pH of the solution was then adjusted to pH 7 with hydrochloric acid and the solution was concentrated to 5 ml by rotary evaporation at 28° prior to elution with water from Sephadex G 25 (fine grade 148 × 2 8 cm) Fractions (2 ml) were collected automatically and were scanned for carbohydrate and for absorbance at 241 *nm* The contents of tubes representing were pooled, concentrated and refractionated as described

Treatment of Oligosaccharides with Sodium Metaperiodate

Solutions (1 0 ml) of certain of the oligosaccharides obtained by treatment of the antigen with acid or alkali were reacted with 0 1 M sodium metaperiodate (0 1 ml) at 22° in the dark and triplicate aliquots (0 1 ml) were withdrawn immediately mixed with 0 1 M ethylene glycol solution (0 1 ml) left for 20 min and frozen Aliquots of the remainder of the reaction mixtures were removed at intervals during 5 hours and were treated similarly The samples were treated with barium carbonate to remove iodate and periodate ions and were centrifuged The solutions were deionized by sequential passage through Dowex 50 (H) resin and Amberlite IR4B (OH) resin prior to analysis for carbohydrate and for HIB activity

RESULTS

Seqential Treatment of Chick Allantoic Antigen with Sodium Metaperiodate and Sodium Borohydride

Component analyses of the antigen before and after this reaction sequence are summarized in Table 1 Approximately 84 per cent of the fucose residues were oxidized, other components apparently resisted oxidation This chemical modification resulted in a 70 per cent reduction of the HIB titre

TABLE 1 *Component Analyses of Chick Allantoic Antigen before and after Sequential Treatment with Sodium Metaperiodate and Sodium Borohydride*

Chemical components	Amount per cent	
	Antigen	'Ox/Red' antigen
D-Galactose	26 0	13 0
Fucose	4 3	0 7
Hexosamine	25 0	25 0
Peptide	13 0	13 0

Treatment with Enzymes

The HIB activity of the antigen was destroyed completely on treatment with crude extracts from a Gram negative coccobacillus The destruction of HIB activity was markedly inhibited by *L*-fucose, but *D* fucose, *D* galactose, 2 acetamido-2 deoxy-*D* glucose and 2 acetamido 2 deoxy *D* galactose had no inhibitory effect Partial fractionation of the glycosidases present in the crude enzyme preparation was achieved by precipitation with ammonium sulphate followed by ion exchange and gelchromatography (14) The HIB titre of the 'oxidized and reduced' antigen was reduced by a further 75 per cent following treatment with two fractions of the crude preparation One of these fraction contained high α *L*-fucosidase activity and low α and β D-galactosidase activities, as shown by reaction with the appropriate p nitrophenyl

The antibody combining activity of the antigen is destroyed by treatment with an enzyme preparation from a soil bacterium (14). The present paper reports further studies of the immunochemistry of the antigen as revealed by HIB tests of fragments obtained from the antigen by treatment with acid, alkali, enzymes and sodium metaperiodate.

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Treatment of Antigen with Enzymes

The enzymes obtained from a Gram negative coccobacillus were purified as described previously (14). Aliquots (0.05 ml) of solutions of the antigen (40 µg/ml) and the oxidized and reduced antigen (500 µg/ml) were mixed with solutions (0.5 ml) of the crude enzyme mixture and of partially purified enzymes derived from it in citric acid-phosphate buffer pH 7 and the solutions were incubated at 30° for 48 hr and then heated at 56° for 10 mins. The solutions and the appropriate

controls were diluted with 0 9 ml PBS pH 7, and the HIB titres were determined These studies were repeated with the incorporation of 0 05 ml of 10 mM solutions of each of the following inhibitors 2 acetamido-2 deoxy *D* glucose 2 acetamido-2 deoxy *D*-galactose, *L* fucose, *D* fucose and *D* galactose The solutions were diluted with 0 85 ml PBS prior to determining the HIB titres

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The HIB activity of the antigen was destroyed completely on treatment with crude extracts from a Gram negative coccobacillus The destruction of HIB activity was markedly inhibited by *L*-fucose, but *D* fucose, *D* galactose 2 acetamido-2 deoxy-*D* glucose and 2 acetamido 2 deoxy-*D* galactose had no inhibitory effect Partial fractionation of the glycosidases present in the crude enzyme preparation was achieved by precipitation with ammonium sulphate followed by ion exchange and gelchromatography (14) The HIB titre of the "oxidized and reduced' antigen was reduced by a further 75 per cent following treatment with two fractions of the crude preparation One of these fraction contained high α *L* fucosidase activity and low α and β *D* galactosidase activities, as shown by reaction with the appropriate p nitrophenyl

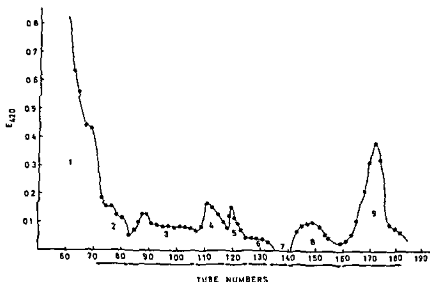


Fig 1 Acid hydrolysis (0 08N hydrochloric acid, 30 min 100°) of components of apparently large molecular weight obtained on hydrolysis of antigen with 0 04N hydrochloric acid, 30 min 100° Hydrolysate eluted from Sephadex G 25

●——● cysteine sulphuric acid analysis for hexose
⟨——⟩ tubes bulked for analysis of peaks designated 1–9

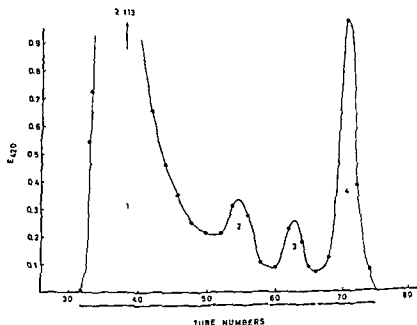


Fig 2 Acid hydrolysis (0 08N hydrochloric acid 30 min 100°) of components of apparently large molecular weight obtained on hydrolysis of antigen with 0 08N hydrochloric acid, 30 min 100° Hydrolysate eluted from Sephadex G 25

●——● cysteine sulphuric acid analysis for hexose
⟨——⟩tubes bulked for analysis of peaks designated 1–4

glycosides, the other fraction contained high β D galactosidase activity and low α D galactosidase and α L-fucosidase activities Treatment with fractions of the enzyme preparation that contained high N acetyl hexosaminidase and very low α L-fucosidase and α and β D galactosidase activities has no effect on the HIB titre of the oxidized and reduced antigen

Hydrolysis with Acid

The initial mild hydrolysis of the antigen liberated only D galactose (3 9 per cent) and fucose (4 1 per cent) as monosaccharides A second slightly stronger, hydrolysis of the material of high molecular weight thus obtained liberated further amounts of *D* galactose (1 5 per cent) and fucose (0 1 per cent) together with oligosaccharides which were partially separated by elution from Sephadex G 25 (Fig 1) Oligosaccharides and more *D* galactose (0 35 per cent) were obtained on further hydrolysis of the material of high molecular weight obtained from the second hydrolysis (Fig 2) The results of gasliquid chromatography (GLC) analysis of neutral and basic carbohydrate components of the

TABLE 2 *Component Analyses of Products Obtained by dilute Acid Hydrolysis of the Antigen*

Acid hydrolysis	Peak No	Analytical data Total amounts in µg*			
		Gal	Fuc	GluN	GalN
	1				
	2	69	—	98	7
	3	685		88	—
Second	4	324		—	—
0 08 N HCl	5	145	16		—
30 min 100	6	54		Trace	Trace
	7	—			—
	8	279	—	133	15
	9	900	57	—	—
	1	+	—	+	+
Third	2	90	—	—	—
0 08 N HCl	3	67	—	—	—
30 min 100	4	200	—	—	—

* expressed as total glycopeptide present

recovered oligosaccharides are given in Table 2

Only the relatively acid resistant core material (peak 1 Fig 2) obtained after three mild treatments of the antigen with acid, and the oligosaccharides (peak 2 and 3, Fig 1) and (peak 2 Fig 2) of apparently high molecular weight showed HIB activity The core material contained D-galactose, 2 amino-2-deoxyglucose and 2 amino 2-deoxy galactose and the same ratios of component amino acids as were found in the intact antigen Its HIB activity was not affected following treatment with sodium metaperiodate The HIB active oligosaccharides contained *D* galactose and 2 amino 2 deoxyhexose (peak 2 and 3 Fig 1) and *D* galactose (peak 2, Fig 2), but no fucose (see Table 2)

Treatment with Dilute Alkali

Treatment of the antigen with 0 2 M sodium hydroxide at 25° resulted in a marked increase in the absorbance of the solution at 241 nm and the liberation of oligosaccharides (Fig 3) together with D galactose (approx 5 per cent) and a small proportion of the fucose content of the antigen Refractionation of products in peak 2–4 (Fig 3) gave three well resolved fractions each of which contained *D* galactose and fucose HIB activity was found in the first of these three fractions and in the alkali resistant core material (peak 1 Fig 3), which contained *D* galactose but no fucose This HIB activity was reduced markedly following treatment with sodium metaperiodate which destroyed approx 50 per cent the galactose and all the fucose residues in the first fraction and some of the *D* galactose in the alkali resistant core material The only other alkali-produced components which showed HIB activity were peaks 5 and 6 (Fig 3) which contained galactose and in peak 5 only, a small proportion of fucose In general the products obtained on treatment of the antigen with alkali showed greater HIB activity than those obtained on treatment of the antigen with acid

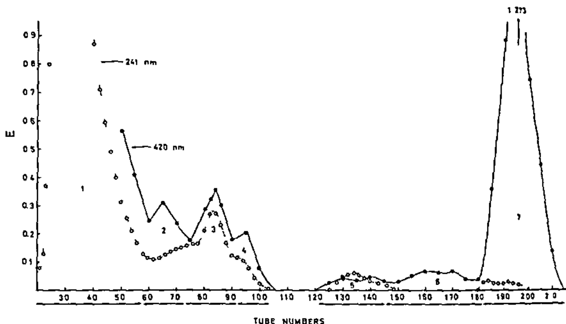


Fig 3 Elution of alkali treated allantoic antigen (0 2N sodium hydroxide 32 hr 25°) from Sephadex G 25

●——● cysteine sulphuric acid analysis for hexose
○- - -○ absorbance at 241 nm
⟨——⟩ tubes bulked for analysis of peaks designated 1 7

DISCUSSION

Considerable information about the structure of this chick allantoic antigen has been obtained from studies using approximate 50 mg of purified antigen Attempts to isolate and characterize fragments of the antigen in order to define the relationship between its structure and immunological properties present a considerably greater problem, using such relatively small amounts of antigen Certain new information has been obtained however from the present work Previous studies have shown (3) that peptide and sialic acid residues are not involved in the HIB active site Confirmation that *L*-fucose residues in the antigen might be essential for HIB activity was obtained in the present work Thus, the crude preparation of bacterial enzyme completely destroyed the HIB activity of the antigen and this destruction was markedly inhibited by *L-fucose* but not by *D* fucose and *D* galactose The importance of *D* galactose residues as well as *L* fucose residues in the HIB active site was shown in studies of the oxidized and reduced antigen obtained by sequential reaction with sodium metaperiodate and sodium borohydride This reaction sequence destroyed 50 per cent of the *D* galactose residues and 81 per cent of the fucose residues with a concomitant reduction in the HIB titre of 70 per cent It is unlikely that fucose residues are solely responsible for the HIB activity of the antigen however since only 16 per cent of such residues are present in the oxidized and reduced antigen whereas 30 per cent of the HIB activity remained Treatment of the oxidized and reduced antigen with enzymes further emphasized the importance of D galactose residues in the HIB active site

HIB active oligosaccharides were produced both by acid and alkali treatment of the antigen It should be emphasized that the products obtained on treatment of the antigen with acid and alkali were essentially repro-

ducible The chemical consequences of these reactions, which have been discussed in detail elsewhere (8, 9, 10), were confirmed Thus mild treatment with acid liberated *D* galactose and fucose from terminal non reducing residues of *D* galactofuranose and fucose respectively Under slightly stronger hydrolysis conditions, HIB active oligosaccharides containing exclusively *D* galactose and hexosamine residues or *D* galactose residues alone were released due to the cleavage of *D* galactofuranosidic linkages located in the interior of carbohydrate chains in the antigen These results confirm the importance of *D* galactose residues as determinants of the HIB activity of the antigen

The carbohydrate peptide linkage regions in the allantoic antigen consist of alkali labile, glycosidic linkages between 2 acetamido 2 deoxygalactose and serine and/or threonine Treatment with dilute aqueous alkali results in β elimination of the carbohydrate which may subsequently undergo further alkali induced reactions and the formation of an α β unsaturated amino acid which results in the increased absorption of the reaction mixture at 241 nm The greater relative HIB activity of the oligosaccharides obtained on treatment with alkali as compared with those obtained on treatment with acid is probably due to the presence of *D* galactofuranose and fucose endgroups in the former products The marked destruction of the HIB activity of certain of the alkali produced fragments by treatment with sodium metaperiodate further confirms the conclusion that *D* galactose residues located in the interior of the antigen are involved in the HIB active site The HIB activity of the *acid* resistant core material however was unaffected by periodate oxidation This indicates that the *D* galactose HIB determinant residues in this core are substitued in a manner which renders them resistant to oxidation by periodate The importance of sulphate and acetyl residues in the immunochemistry of the antigen remains to be investigated

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Acta path microbiol scand Section B 79, 357-364, 1971

A COMPARISON OF PHAGE PATTERN AND ANTIGENIC STRUCTURE WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM CATTLE

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The majority of the strains could be identified by their biochemical properties as belonging to the bovine variant of *Staph aureus* The carrier strains differed from the mastitis strains both biochemically, by phage typing and by serological typing Within each group the strains were very homogeneous The significance of the homogeneity and of the particular biochemical properties of the carrier strains are discussed

Staphylococci are widely disseminated in man and in various domestic animals Definable host adapted variants of *Staphylococcus aureus* no doubt exist, *e g* in man and in cattle Our knowledge of the characteristics of the hitherto known variants is, however, not complete Neither has evidence been presented as to the constancy of the individual characteristics of the variants under different living conditions The differentiation and classification of the host adapted variants is at present both a practical and a theoretical problem

A complete biochemical examination is of prime importance in the type differentiation (22, 16, 17, 12, 19 23, 25, 5) Phage typing (3, 12, 18) and serological typing (11, 26, 13, 25) have also proved useful in the differentiation of *Staph aureus* strains of human and bovine origin Considerable diagnostic difficulties may, however, arise when strains are transmitted through contact between man and animal or between different animal species

The present report contains a study of staphylococci isolated from bovine mastitis and from the nasal cavities of healthy cattle

MATERIALS AND METHODS

From different regions of Bohemia and Moravia 92 strains of *Staph aureus* were obtained from cases of acute bovine mastitis In addition 81 strains

Received 24 ix 70

were isolated from nasal swabs of 667 healthy cows from different agricultural cooperatives of the Olomouc region All the 173 strains were tested simultaneously for biochemical properties phage pattern and antigenic structure

The results of the biochemical examination have been reported by *Hájek* & *Maršálek* (5) The following activities were tested mannitol fermentation coagulation of human and bovine plasma production of the clumping factor hemolysins fibrinolysin pigment and the egg yolk factor, the type of growth on crystal violet agar the sensitivity to sublimate and to penicillin and a number of other antibiotics

Phage typing was carried out according to the method of *Blair* & *Williams* (1) All strains were tested with the phages of the International basic set and with phages recommended by *Davidson* (3) for the typing of bovine strains according to the composition of 1966 A number of other phages were also used (Table 2 Table 3) Strains not typable with the routine test dilution (RTD) were tested with RTD × 100 and RTD × 1 000

The serological typing was performed according to the method of *Oeding* (20) The factor sera (a_4 a_5 b_1 c_1, e h_1 h $i_1 t$ $k_2 k_4$ k_1 m n 263 1 263 2) were prepared by immunization of rabbits with human strains of *Staph aureus* and subsequent absorptions Agglutination was performed on slides For technical details see also *Haukenes* (7) The precipitinogens were determined by the double diffusion technique in agar gel according to *Haukenes* & *Oeding* (8) The strains were tested with undiluted rabbit antisera prepared against the human *Staph aureus* strains Wood 46 Cowan I 1503 and 263 Polysaccharide A polysaccharide 263 antigen D and protein A were included as references

RESULTS OF BIOCHEMICAL EXAMINATION

The 173 *Staph aureus* strains isolated from cows were divided on the basis of their biochemical properties into four relatively homogeneous groups (Table 1) The first group comprises 82 strains isolated from cases of bovine mastitis These strains coagulated bovine plasma within 3 hr and human plasma already after 1 hr All were fibrinolysin negative and produced beta hemolysin The majority also produced the egg yolk factor After a massive inoculation on crystal violet agar they showed an orange growth

The second group consisted of 68 *Staph aureus* strains isolated from the nasal cavities

TABLE 1 *Division of the Strains According to Biochemical Properties*

	Mastitis			Nares		
	Bovine	Human	Intermed	Bovine	Human	Intermed
Human plasma 1 hr	66	–	4	3		
Human plasma 3 hr	16	2	4	65	6	5
Bovine plasma 1 hr	48	–	1		–	
Bovine plasma 3 hr	19	–	2	2		
Bovine plasma 24 hr	15	–	1	66	–	2
Clumping factor	79	2	8	67	6	4
Hemolysin alpha	48	1	3	22	–	2
Hemolysin beta	82	2	8	68	7	4
Hemolysin delta	74	2	7	66	7	5
Fibrinolysin	–	2	4		7	2
Pigment orange	74	2	5	67	2	5
Pigment cream	8	–	3	1	–	1
Crystal violet test orange	77		6	2	–	
Crystal violet test violet	5	2	2	66	7	6
Egg yolk factor	64	1	6	6	7	4
Penicillin resist	6	1	–	2	7	–
Sublimate resist	5	2		2	7	2
Total	82	2	8	68	7	6

of healthy cows Also these strains were fibrinolysin negative and formed beta hemolysin but they coagulated bovine and human plasma more slowly and the egg yolk factor was usually not produced The most obvious biochemical difference from the strains of the first group was that all carrier strains except two grew on crystal violet agar with a violet colour

Nine strains had qualitatively quite different biochemical properties and were placed in a third group designated human strains Two of these strains were isolated from mastitis and 7 from the nasal cavities They did not coagulate bovine plasma after 72 hr were fibrinolysin positive resistant to antibiotics and sublimate and were strong producers of the egg yolk factor

Fourteen strains 8 from mastitis and 6 from the nasal cavities could not be classi

TABLE 2 *Phage Typing with the International Basic Set*

Group	Phage	Mastitis			Nares		
		Bovine	Human	Intermed.	Bovine	Human	Intermed.
I	29	–/2	–	–	22/1	–/7	2/–
	52	–	–	–	1/–	–/1	1/–
	52A	–	–/1	–	3/2	–/7	1/–
	79	–/1	–/1	–	3/2	–/6	–
	80	–/1	–	–	35/2	–/6	2/–
II	3A	–/3	–	–	–/1	–	–
	3C	–	–	–	–/1	–	–
	55	–/6	–	1/–	–/1	–	–
	71	–/1	–	–	–/1	–	–
III	6	5/5	1/–	–	–/1	–	–
	42E	46/8	–/1	4/–	1/1	–/7	–
	47	5/4	1/–	–	–/1	–	–
	53	5/–	–	–	–	–	–
	54	11/8	1/1	2/–	1/2	–/4	–
	75	5/–	1/–	–	–	–	–
	77	4/5	–/1	2/–	1/2	–	–
	83A	6/1	1/–	1/–	–	–	–
	84	1/2		1/	–	–	–
	85	–	–	–	–	–	–
IV	42D	58/5	–	8/–	–/1	–	–
M	81	1/4	–/1	–	–/1	–/3	–
	187	–	–	–	–	–	1/–
Add	812		–	–	–	–	–
	825	–	–	–	–	–	–
NT			–	–	26	–	3
Total		82	2	8	68	7	6

RTD/1 000 × RTD

fied satisfactorily by biochemical methods They corresponded in some properties to the strains of the two first groups and in other properties to those of the third group and were designated as "intermediate" strains

RESULTS OF PHAGE TYPING

All the 82 mastitis strains of the first biochemical group were typable with phages both of the International basic set and of the *Davidson* set, usually at RTD In the examination with the basic set the majority of the strains were sensitive to phages 42E and 42D and thus belonged to phage groups III and IV (Table 2) Single reactions were observed with other group III phages at RTD and RTD×1,000 These strains also showed a uniform pattern on examination with the *Davidson* set They reacted with all or most of the 7 phages of group IV, only a few strains giving additional reactions with phages of group III (Table 3)

Of the 68 carrier strains belonging to the second biochemical group 42 gave strong reactions with phages of the International basic set These strains were lysed by phage 80 and/or 29 of phage group I. Twenty-six strains gave only + or ± reactions and were, in accordance with international custom,

TABLE 5 *Precipitinogens*

	Mastitis		Nares	
	No	%	No	%
Polysacch A	91	99	80	99
Polysacch 263	15	16	10	12
Antigen D	81	88	78	96
Protein A	38	41	20	25
Total	92	100	81	100

DISCUSSION

On biochemical examination of the present material of 173 strains of *Staph aureus* isolated from cattle 9 strains were definitely shown to have the characteristics of the human variant whereas 14 strains were intermediate between the human and the bovine variant The remaining 150 strains were considered to belong to the bovine variant (Meyer (17, 19)) They produced beta hemolysin but not fibrinolysin coagulated bovine plasma and were sensitive to antibiotics and sublimate The group consisting of 68 bovine strains isolated from the nares of healthy cattle behaved somewhat differently in the biochemical examination as compared to the 82 bovine mastitis strains The carrier strains coagulated human and bovine plasma more slowly and produced the egg yolk factor less frequently (10 versus 77 per cent) than the mastitis strains and each group produced different types of colonies on crystal violet agar The significance of these biochemical differences is discussed below

The results of phage typing and serological typing conformed well with the grouping of the strains performed on a biochemical basis Each of the typing methods clearly showed that the bovine strains of the mastitis group and those of the carrier group were different The two groups were remarkably homogeneous on phage and serological typing The mastitis strains reacted chiefly with phages of group IV and group III whereas the carrier strains reacted with phages of group I Of the International basic set it was mainly phage 42D and phage 42E that were active against the mastitis strains and phage 80 and phage 29 against the carrier strains With the *Davidson* set a wider range of phages was active against the mastitis strains whereas the carrier strains reacted predominantly with phage AC_1

Serologically the c_1 agglutinogen was characteristic of the mastitis strains and the h agglutinogen of the carrier strains In 83 per cent of the typable mastitis strains the c_1 agglutinogen was present the h_2 agglutinogen being found in 97 per cent of the typable carrier strains These antigens were also most common in a material of bovine strains examined by *Marandon & Oeding* (13) This may indicate that the c_1 and h_2 antigens are relatively characteristic of bovine staphylococci but further investigation is required

Of the mastitis strains 73 per cent were typable serologically and of the carrier strains all except one were typable Altogether type agglutinogens were demonstrated in 127 of 150 strains of the bovine variant (85 per cent) This is a favourable result compared with the majority of previous reports on serological typing of bovine strains (see 13)

The homogeneity of each of the two materials of bovine *Staph aureus* strains demonstrated both biochemically on phage typing and on serological typing is remarkable According to other investigations (11 26) a greater variation in phage patterns and serological types might have been expected The homogeneity is the more remarkable as the strains were collected from various geographical regions It seems that at the time of isolation few bovine *Staph aureus* strains were in circulation in the regions involved The circumstance that our two materials were obtained exclusively from bovine mastitis and from the nares of healthy cattle respectively may also have contributed to the result

The significance of the biochemical differences in the carrier strains as compared to the usual bovine variant here represented by the mastitis strains is not easy to evaluate Further experience will show whether the differences are accidental or whether they

justify the establishment of a new variant The biochemical characteristics of the carrier strains may be associated with special environmental conditions on the mucous membranes of healthy cattle The question of whether there is a connection between the biochemical properties and the virulence of these carrier strains is also a matter for discussion (*Hajek* & *Maršálek* (5)) This problem has also been discussed with regard to human staphylococci (4 10, 9, 6) So far, however, few materials of staphylococci isolated from the nose of healthy cattle have been examined It seems that only a few per cent of cattle have *Staph aureus* in their nasal cavities and then in small numbers (24, 2 15 21, 14, 25) This is in accordance with our findings (5) but in contrast to a carrier percentage of 67 reported by *Pulverer* & *Entel* (23)

The phage typing was performed by Dr H *Meyer* Institut fur expermentelle Epidemiologie — Zentrallaboratorium fur Lysotypie — Wernigerode DDR We are much indebted to Dr *Meyer* for his valuable contribution

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Serologically the c_1 agglutinogen was characteristic of the mastitis strains and the h_2 agglutinogen of the carrier strains In 83 per cent of the typable mastitis strains the c_1 agglutinogen was present, the h_2 agglutinogen being found in 97 per cent of the typable carrier strains These antigens were also most common in a material of bovine strains examined by *Marandon & Oeding* (13) This may indicate that the c_1 and h_2 antigens are relatively characteristic of bovine staphylococci, but further investigation is required

Of the mastitis strains 73 per cent were typable serologically and of the carrier strains all except one were typable Altogether type agglutinogens were demonstrated in 127 of 150 strains of the bovine variant (85 per cent) This is a favourable result compared with the majority of previous reports on serological typing of bovine strains (see 13)

The homogeneity of each of the two materials of bovine *Staph aureus* strains demonstrated both biochemically on phage typing and on serological typing is remarkable According to other investigations (11 26) a greater variation in phage patterns and serological types might have been expected The homogeneity is the more remarkable as the strains were collected from various geographical regions It seems that at the time of isolation few bovine *Staph aureus* strains were in circulation in the regions involved The circumstance that our two materials were obtained exclusively from bovine mastitis and from the nares of healthy cattle, respectively may also have contributed to the result

The significance of the biochemical differences in the carrier strains as compared to the usual bovine variant, here represented by the mastitis strains is not easy to evaluate Further experience will show whether the differences are accidental or whether they

Acta path microbiol scand Section B 79 365-371, 1971

DEMONSTRATION OF ANTIBODIES TO MUSCLE BY ABSORPTION OF RHEUMATOID FACTOR

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Samples of skeletal muscle tissue were treated with normal human serum and with sera from 25 patients with myasthenia gravis Rheumatoid factor-containing serum was absorbed with the washed tissue sediments Waaler's test was performed before and after the absorption Tissue treated with normal serum did not reduce the titre of rheumatoid factor Tissue treated with sera from 21 patients with myasthenia gravis gave a significant titre fall Four sera from myasthenia gravis patients contained rheumatoid factor Tissue treated with these sera did not bind isologous rheumatoid factor When the sera were treated with mercaptoethanol before incubation with the tissue sediments absorpt on both of isologous and of autologous rheumatoid factor was demonstrated Thus absorption of rheumatoid factor allows discrimination between γ globulins bound to muscle tissue by the Fab portion or the Fc portion

Normal sera contain muscle binding γ globulins which combine with the tissue *in vitro* by the Fc fragment (*Aarli* 1970) Serum γ globulins from patients with myasthenia gravis however, combine specifically with skeletal muscle and thymus tissue by the Fab piece This type of reaction suggests that an antigen antibody reaction has occurred In an antigen antibody combination the γ globulin molecule is supposed to undergo structural changes (*Milgrom Dubiski & Wozniczko* 1956) Rheumatoid factor combines more avidly with structurally altered than with native human γG globulin Accordingly, rheumatoid factor has been regarded as an antibody to altered human γG antibodies (*Aho & Simons* 1963) In myasthenia gravis only muscle antibodies of γG globulin class have been described (*McFarlin et al* 1968, *Aarli & Tönder* 1970) If rheumatoid factor can react with muscle tissue treated with myasthenia gravis serum, this would strongly support the assumption that the γG globulins had combined with the tissue in an antigen-antibody reaction This paper presents observations on the reactivity of sensitized muscle tissue sediment with rheumatoid factor

Received 22 ix 70

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MATERIALS AND METHODS

Tissue Preparations

Normal human skeletal muscle tissue was obtained and prepared as described earlier (*Aarli & Tonder* 1970)

Sera

The material comprised sera from 24 patients suffering from myasthenia gravis (MG) Muscle binding γ globulins were demonstrated in increased

amounts in 12 patients ("sero positive"), and in amounts corresponding to that of normal sera in 12 ('sero-negative') The results of earlier studies with these sera have been described (*Aarli & Tonder 1970, Aarli 1970*) Because of limited availability, serum MG 4 could not be included in this study

In addition 2 MG sera, containing rheumatoid factor (RF), were kindly provided by *B Vandvik*, M D, Department of Neurology, Rikshospitalet, Oslo (serum A S) and *J P Hesselberg*, M D, Department of Neurology, Trondheim Central Hospital, Trondheim (serum I G)

Normal sera were obtained from healthy blood donors

Sera from patients suffering from rheumatoid arthritis were obtained from Hatlestad Hospital and the Norwegian Women's Health Organization's Hospital for Rheumatic Diseases Haugesund

Treatment with Mercaptoethanol

This was performed according to *Yokoyama & Fudenberg (1964)*, except that isotonic phosphate buffered saline, pH 7 2 (saline) was used instead of saline borate buffer

γ globulin

Human γ globulin (Fraction II), 16 5 per cent solution, was purchased from AB Kabi, Stockholm Sweden

Denatured γ globulin was prepared by heating a 1 per cent solution at 63°C for 10 min

Pepsin digestion of γ globulin was performed as described earlier (*Aarli 1970*)

Red Cells

Sheep red cells from one animal and human O $R_1 R_2$ red cells from one individual were obtained and prepared as described earlier (*Aarli & Tonder 1970*)

Antiglobulin Consumption Test (AGCT)

This was performed as described earlier (*Aarli & Tönder 1970*)

Test for RF (Waaler's test)

The test was essentially carried out as described by *Tonder & Milgrom* (1964) However, the sheep red cells were sensitized by antiserum at a dilution four times higher than the agglutination titre as determined after centrifugation of the tubes at 1,000 g for 1 min The sera to be examined were absorbed with unsensitized sheep red cells Two fold dilutions in saline were prepared in 0 2 ml volumes Two-tenths of a millilitre of a 0 5 per cent suspension of sensitized red cells were added to each tube The racks were shaken and left at room temperature for 1 hr After thorough shaking, the tubes were centrifuged at 1,000 g for 1 min Agglutination was recorded after gently agitating each tube The clumping was graded at the moment when all erythrocytes were dislodged from the bottom The titre was recorded as the reciprocal of the highest serum dilution giving at least 1 + reaction

Absorption of RF

Thirty mg of lyophilized muscle tissue was suspended in 10 ml of saline and centrifuged for 5 min at 1 000 g The washing was repeated twice The sediment was resuspended in saline and 2 ml MG serum (or as indicated) was added Sediment incubated with saline followed as control The suspension was incubated for 20 min at 37°C and then centrifuged at 1,000 g for 10 min The sediment was washed 4 times in 10 ml of saline and then resuspended in 0 6 ml of rheumatoid arthritis serum (RAS) diluted 1 64 (RF titre 1024) The mixture was shaken and incubated at 4°C overnight and then at room temperature for 1 hr After centrifugation at 1 000 g for 10 min the supernatant was withdrawn and tested for RF activity Careful removal of the supernatant was very important as small tissue particles interfered with the results A control with saline instead of RAS was therefore always included

In some experiments two or more successive absorptions were performed

Elution of RF from Sensitized Sediments

Autologous RF Samples of 30 mg tissue were incubated with 2 ml MG serum as described Sediments treated only with normal serum or with saline followed as control After washing and centrifugation at 1,000 g for 10 min each of the samples was suspended in 1 5 ml 0 1 M acetate buffer, pH 4 1 kept at room temperature and shaken at intervals After 30 min the tubes were centrifuged at 1,000 g for 10 min and the supernatant carefully withdrawn The sediments were then once more suspended in 1 5 ml of the acetate bufffer incubated and centrifuged as before and the two supernatants were pooled and dialyzed at 4°C for 24 hrs against saline

Isologous RF Samples of 30 mg muscle tissue were treated with MG serum as described Controls included saline and normal serum After centrifugation and washing, 1 ml of a RA-serum diluted 1 4 (titre 512) was added The tubes were incubated at 37°C for 1 hr and overnight at 4°C centrifuged and washed three times The sediments were thereafter treated twice with acetate buffer as described and the supernatants pooled and dialyzed

The eluates were then absorbed with unsensitized sheep red cells before use in Waaler's test

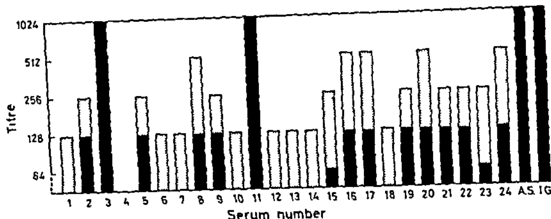


Fig 1 Titres of samples of one RAS after absorption with muscle tissue sensitized with each of 25 MG sera Titres after one absorption are indicated with dotted columns Black columns Two or more absorptions performed Titre of serum absorbed with tissue sediment alone 1024

Inhibition of Agglutination

was performed according to *Natvig & Tonder* (1967)

Preparative Ultracentrifugation

Eluates were separated in a 10–40 per cent sucrose gradient by centrifugation for 17 hrs in a Spinco Model L/L2 ultracentrifuge at 35 000 rpm (*Kunkel* 1960) Sixteen successive fractions were obtained from a pin hole just above the bottom of the tubes The fractions, numbered from bottom to top were tested without further treatment

EXPERIMENTS AND RESULTS

Absorption of Isologous RF

Twenty-five portions of muscle tissue sediment were sensitized respectively with 25 MG sera To each sample was then added RAS as described After incubation and centrifugation, the supernatants were used in Waaler's test (Fig 1) A reduction of titre was observed with most sera Because of the errors involved in the absorption and titration procedures, a decrease in titre corresponding to one step in the doubling dilutions was considered insignificant A decrease corresponding to two steps was interpreted as doubtful absorption effect while a decrease corresponding to three steps was considered significant

With 4 sera, no titre reduction occurred even with repeated absorptions With 8 sera, which all belonged to the group of "seropositive", one absorption resulted in a significant titre reduction With 13 sera, 12 of which were "seronegative", the titre was reduced by 1 of 2 steps After 2 absorptions, however, the titres were reduced significantly

Effect of Autologous RF in Sera

Absorption with tissue sensitized with 4 sera MG-3, MG-11, A.S and I.G did not reduce the RF activity However, all 4 sera gave a pathological consumption when tested in the AGCT (MG-3 128, MG 11 64, A S 32, and I G 64) and should therefore, contain muscle binding γ globulin in increased amounts

Presumably, the tissue bound γ-globulin from the sera combined with another factor, inhibiting the binding of the added RF All 26 MG sera were therefore tested for RF activity With the 4 sera in question, increased RF titres were recorded (Table 1) The remaining sera gave titres less than 64, which was considered to be within normal limits

The four sera were treated with mercaptoethanol to destroy autologous RF and then used for sensitization of muscle tissue The same sera, not exposed to mercaptoethanol but otherwise treated similarly, were used as controls The sensitized tissues were thereafter used in absorption experiments with RAS as described A significant RF titre

TABLE 1 *RF Titres of 4 MG Sera before and after Absorption with Skeletal Muscle Tissue Incubated with the Corresponding Serum Effect of Mercaptoethanol Treatment of the Sera before Incubation with Tissue*

	Serum number			
	MG 3	MG 11	I G	A S
Unabsorbed sera	2048	1024	512	256
Absorbed with tissue incubated with				
whole serum	2048	1024	512	256
ME treated serum	<128	<64	128	32

reduction was found with 3 sera and a doubtful reduction with the 4th No fall in titre was recorded with the control sera Accordingly binding of isologous RF also occurred with these sera but only after inactivation of the autologous RF

Absorption of Autologous RF

The 4 RF containing MG sera were treated with mercaptoethanol and the products used for sensitization of muscle tissue To each sediment was added 0 6 ml of the same MG serum which was used for sensitization but this time not treated with mercaptoethanol With each of the samples, the MG serum added was diluted 16 times lower than its RF titre dilution After incubation the tubes were centrifuged and the supernatants tested for RF activity With 3 sera a significant titre reduction occurred The RF titre of serum I G was reduced by a factor of 4 (Table 1)

Two sera from patients with rheumatoid arthritis not suffering from myasthenia gravis were then tested in a similar way They gave no pathological consumption in the AGCT with skeletal muscle tissue When tested for absorption of autologous rheumatoid factor as described for the MG sera no titre reduction was observed

Elution of RF from the Sediments

Eluates were first prepared from two samples of muscle tissue treated with serum MG 3 and MG 11 The eluates agglutinated sheep red cells with titres of 16 and 8, respectively The reaction was abolished after prior mercaptoethanol treatment and was also inhibited by denatured γ globulin

Eluates prepared from muscle tissue sediments treated with serum MG 12 and MG 24 thereafter with RAS also agglutinated red cells to titres of 4 After fractionation by ultracentrifugation the reactivity was found in the mercaptoethanol sensitive γM fractions Eluates prepared from tissue sensitized with normal serum or saline before incubation with RAS gave no agglutination in Waalers test

Controls

Whether lyophilized muscle tissue in itself could absorb RF was investigated in the following experiment Samples of 30 60 and 120 mg washed muscle tissue (dry weight) were incubated with RAS and the supernatants thereafter used in Waalers test No titre reduction was observed, indicating no binding of RF to unsensitized muscle tissue

In order to investigate the effect of incubation with normal human serum 4 samples of 30 mg muscle tissue were treated with 1 2, 4 and 8 ml respectively of fresh and of inactivated serum from 5 healthy individuals The tissues were then incubated with RAS There was no decrease in RF activity Furthermore with samples from a pool of sera from 20 healthy individuals no absorption of RF could be demonstrated Two repeated absorptions did not result in significant titre fall Accordingly normal γ globulin bound to muscle tissue do not combine with RF

Samples of 30 mg muscle tissue were then incubated with 4 ml of a one per cent γ-globulin solution with the same amount of heat denatured γ globulin and with saline respectively The samples were used for absorption of RAS and the supernatants tested as described Tissue incubated with untreated γ globulin gave no titre reduction With tissue incubated with heat denatured γ globulin a fall in titre occurred and one repeated absorption reduced the titre significantly

TABLE 2 *AGCT Performed with Muscle Tissue Incubated with Native and Denatured γ Globulin before and after Pepsin Digestion*

Muscle tissue incubated with	Titre of Antiserum after absorption	Antiglobulin consumption
Saline	256	2
Native γ globulin	32	16
F(ab) native γ globul n	256	2
Denatured γ globulin	32	16
F(ab)₂ denatured γ globulin	256	2

In order to investigate the binding of denatured γ globulin to the muscle tissue AGCT was performed with samples of 10 mg of muscle tissue incubated with 2 ml of a one per cent solution of γ globulin native and denatured before and after pepsin digestion Controls were muscle tissue incubated with saline and with γ globulin not exposed to enzyme but otherwise treated as the digested samples To the sensitized tissue sediments were added 0 5 ml of antiglobulin serum (titre 512) diluted 1 16 The results are given in Table 2 Tissue sensitized with F(ab) fragments prepared from native or denatured γ globulin did not reduce the titre of antiserum more than untreated tissue (basal consumption) Accordingly, neither native nor denatured γ globulin combined with muscle tissue after pepsin digestion

DISCUSSION

When samples of muscle tissue were treated with MG sera and RAS was added to the sediment a reduction of the RF activity in the supernatant occurred with 21 out of 25 patient sera Although two absorptions were necessary with 13 of the sera in order to obtain a significant fall in titre no titre reduction was observed even after three absorptions with normal sera Furthermore eluates prepared from the MG RAS sensitized sediments agglutinated sensitized sheep red cells in Waaler s test This was due to a factor which sedimented with the γM globulins and exhibited characteristics compatible with RF The results presented have thus established that RF combines with tissue bound γ globulin from MG sera Earlier published data (*Aarli* 1970) indicate that the muscle binding γ globulins combine with the tissue by the Fab fragment Accordingly, this Fab binding induces the structural changes in the Fc part of the molecule essential for reaction with RF

With 4 MG sera however, no reduction of the RF titre occurred Each of these sera contained increased amounts of RF Presumable the γG antibodies bind autologous RF after they have reacted with the muscle tissue This complex will be more or less saturated with RF preventing a further uptake of RF from the added RAS This assumption was supported by the results of mercaptoethanol treatment of the sera prior to incubation with tissue Mercaptoethanol causes γM RF to dissociate into inactive monomers while the γG antibodies remain intact Muscle tissue incubated with such sera absorbs RF similar to muscle tissue incubated with MG sera which do not contain RF

Inhibition of absorption by autologous RF indicated autoreactivity of the RF This was further verified by the demonstration of RF in eluates prepared from muscle tissue sensitized with two of these sera It was therefore not surprising that absorption of autologous RF could be demonstrated with the four sera in question These reults clearly demonstrate autoreactivity between tissue bound muscle antibodies and RF from the same individual

Relevant observations have been reported in other conditions Thus *Kunkel et al* (1958) described positive serological reactions for RF in patients with sarcoidosis and other hyperglobulinemic states RF like antibodies also occur after prophylactic immunization with tetanus or diphteria toxoid (*Aho Somer & Salo* 1967) and even transiently in association with antibody response to infectious disease (*Svec & Dingle* 1965) Furthermore

Aho et al (1961) reported absorption of RF by both auto- and isologous diphteria toxoid antitoxoid precipitates In addition, anti-γ-globulin factors resembling human RF can be produced in rabbits after immunization with antigen/antibody complexes (*Williams & Kunkel* 1963) The assumption that RF is an auto-antibody to altered γ globulin is therefore supported by both clinical and experimental data It is tempting to assume that, in the 4 MG-sera in question, RF appeared as a response to the muscle antigen(s)/antibody complexes and that the reactant counterpart to RF is γG globulin bound to muscle tissue However, the observations hardly allow definite conclusions, and RF in MG sera may also have been provoked by some other process independent of the muscle antibody production In any case, some unknown factor must be involved, since only 4 out of the 26 MG-sera contain RF in significantly increased amounts while all sera contain muscle antibodies

Muscle binding γ globulins are also present in normal human sera The data show, however, that RF does not combine with muscle tissue treated with such sera Even when larger serum volumes were employed or the absorptions repeated, no significant reduction of the RF titre occurred This is in accordance with the observation that pepsin digested normal γ globulin does not bind to muscle tissue (*Aarli* 1970), and is taken as additional evidence that the γ-globulin combines with the tissue by the Fc portion of the molecule Presumably this binding does not lead to the necessary structural changes in the Fc piece

On the other hand, tissue incubated with heat-denatured γ-globulin combined with RF But the reaction was not dependent on an antigen/antibody reaction, since pepsin digested denatured γ-globulin did not combine with muscle tissue Accordingly denatured γ-globulin also binds to the tissue by the Fc portion of the molecule and the structural changes induced during denaturation will allow a combination with RF

In addition to binding to muscle tissue, the Fc region of the γ-globulin molecule is essential for a series of serological activities other than antibody function Thus skin fixation in guinea pigs (*Ovary & Karush* 1961), entry of antibodies through the mouse gut (*Morris* 1963) and through foetal membranes in rabbits (*Brambell et al* 1959) are dependent on the Fc piece These functions may represent a general modus of non antibody binding to tissue of which binding to muscle tissue might represent one example Possibly this binding represents part of a general defense mechanism of tissue cells where γ-globulins are fixed to the cells by the Fc portions of the molecule, while the Fab region is free to react with specific antigens

This study was aided by a grant from *L Meltzers hoyskolefond* I am indebted to Mrs *F Holst* for technical assistance

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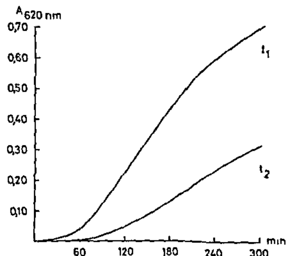


Fig 2 Time Course of Egg Yolk Reaction at Different Temperatures

To 1 0 ml crude filtrate was added 0 8 ml 0 1 M tris buffer pH 7 6, containing 0 130 M NaCl and 0 010 M $MgCl_2$, and 0 2 ml egg yolk solution During the incubation (t_1 37°C, t_2 26°C) optical *densities were read every 30 min in a Beckman* Spinco 151 Spectrocolorimeter, at 620 nm, against blanks

Temperature quotient, $Q_{10\,C}$ =

$$\frac{\text{K value } t_1 \times 10}{\text{K-value } t_2 \times (t_1 - t_2)} \approx 1\,9$$

TABLE 1 *Influence of Ions on Egg Yolk Reaction*

Ion, mmol/l	Per cent activity
Mg^{++}, 5	170
, 10	154
, 20	123
Ca 5	10
Zn^{++}, 5	0
Control	100

To 0 8 ml crude filtrate was added 0 8 ml 0 1 M tris buffer pH 7 6, containing 0 130 M NaCl, 0 2 ml ion solution (chloride salts, ten times final concentrations) in 0 145 M NaCl, and finally 0 2 ml egg yolk solution Incubation (37°C) and readings were carried out as described in Fig 2

During the incubation period, total phosphorus in the trichloracetic filtrate increased from 1 4 to 9 2 μg/ml, inorganic phosphorus in the filtrate remained virtually unchanged (1 1 and 1 2 μg/ml), and phosphorus in the lipid fractions disappeared, as demonstrated

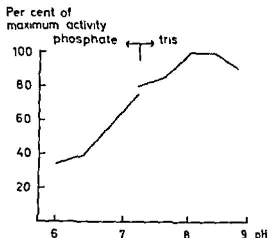


Fig 3 Influence of pH on Egg Yolk Reaction

To 1 0 ml crude filtrate was added 0 2 ml egg yolk solution and 0 8 ml buffer In the pH range 6 0–7 2 a 0 03 M phosphate buffer containing 0 090 [illegible] range [illegible] M N [illegible] and [illegible] 2

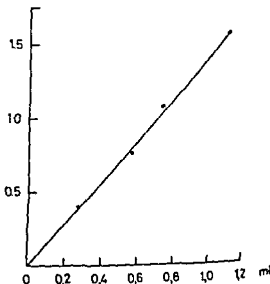


Fig 4 Calibration Graph of Egg Yolk Reaction

Crude filtrate in portions of 0 275, 0 55, 0 725 and 1 1 ml was mixed with 0 8 ml 0 1 M tris buffer pH 7 6, containing 0 130 M NaCl and 0 010 M $MgCl_2$, 0 1 ml egg yolk solution and 0 145 M NaCl, up to 2 0 ml Incubation 37° C) and readings were carried out as described in Fig 2

TABLE 2 *Heat Inactivation of Egg Yolk Reaction (Per Cent of Initial Activity)*

Temperature °C	Duraton of incubation period, mins 5	15	30	90
22	100	100	100	100
37	100	100	100	96
45	88	61	21	0
52	0			

Portions of 1 0 ml crude filtrate were heated in water baths then rapidly cooled in ice water followed by addition of 0 8 ml 0 1 M tris buffer pH 7 6 containing 0 130 M NaCl and 0 010 M MgCl and finally 0 2 ml egg yolk solution Incubation (37°C) and readings were carried out as described in Fig 2

by spraying the thin layer plates with molybdenum spray

The turbidity of the reaction mixture increased during the incubation period Time and temperature of incubation influenced the development of turbidity as shown in Fig 2 After an initial lag period a rectilinear increase in optical density could be observed for approximately 150 min In the interval 26–37°C the temperature coefficient (Q_{10}°C) was 1 9 The development of turbidity was accelerated by Mg and inhibited by Ca (Table 1) Addition of 0 005 M Zn^{++} resulted in precipitation of the egg yolk solution and complete inhibition of the reaction Optimum pH of the egg yolk reaction was found to lie between 8 and 8 4 (Fig 3) The reaction was unaffected by changes in egg yolk concentration between 2 5 and 15 per cent while a linear relationship existed between filtrate concentration and the reaction velocity (Fig 4) Preheating of crude filtrate for 5 min at 52°C resulted in inactivation (Table 2)

Haemolytic and turbidity forming activities behaved identically when examined by gel filtration Both were excluded by Sephadex G 25 coarse (Fig 5a) and partly excluded by G 50 coarse showing similar elution patterns in G 100 and in G 200 (Fig 5b)

Electrophoresis showed the haemolytic and turbidity forming components to have similar running characteristics (Fig 6)

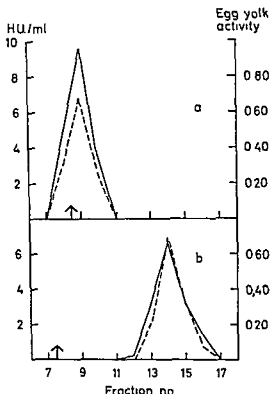


Fig 5 Egg Yolk Turbidity and Haemolytic Activity Examined by Gel Filtration

Portions of 2 ml crude filtrate were applied to Sephadex columns and eluted with 0 05 M tris buffer pH 7 2 containing 0 130 M NaCl Bed volume were 24 5 × 1 5 cm sample and fraction volumes 2 ml G 25 coarse (a) flow rate 27 ml/cm²/hr pressure 25 cm H O G 200 (b) 3 5 ml/cm²/hr 12 cm H O ↑ Void volume, estimated by 0 1 per cent Blue Dextran 2000 Haemolytic activity was examined employing human red cell suspensions as previously described (6) Egg yolk turbidimetry was performed by adding 0 1 ml egg yolk solution and 0 5 ml 0 1 M tris buffer pH 8 8 containing 0 130 M NaCl and 0 20 M $MgCl_2$ to 1 5 ml portions of the fractions (final pH 8 05) Incubation (37°C) and readings were carried out as described in Fig 2

——— Haemolytic activity — — — Egg yolk activity

DISCUSSION

The splitting of PC and PE indicates the presence of a phospholipase The formation of what appears to be a diglyceride is accompanied by an increase in the organically bound fraction of acid soluble phosphorus This could be explained by the presence of

that of phage 84 nor 85, and strains lysed by 84, 85 and 6557' in various patterns seemed to differ with respect to antibiotic-resistance

In recent years a new wave of mostly non-typable methicillin-resistant epidemically occurring strains has followed, and the Danish staphylococcal laboratory has found a new experimental phage (592')[1] useful in typing them, evidence suggests that these strains also belong to the complex which comprises strains lysed by the phages 83A, 84, 85 and 6557'

By re-typing staphylococcal bacteraemia strains, preserved from the years 1962 68, with the phages 84, 85, 6557' and 592' it has been possible to follow the various phage-patterns back through the years and to register their occurrence and spread to the hospitals

Furthermore, it is the aim of the present report to give experimental evidence which would suggest relationship among strains of various phage-patterns included in the complex and to correlate the patterns with resistance to antibiotics

MATERIAL AND METHODS

Strains 2237 *Staphylococcus aureus* strains isolated from 2162 cases of septicaemia during the years 1962–1968 Part of the material has previously been made use of as described by *Jessen et al* (1969)

Experimental typing phages 6557' was isolated and described by *Bulow* (1968 a) 592 was isolated as a variant of phage 84 and propagated on a non typable strain (P S 592) It belongs to serological group B The lytic spectrum given in Table 1 is not identical with any of those of the phages 84, 85, D, 6557 (*Bulow* 1968 a)

Used at RTD only phage 592 was applied to various strains lysed by internationally recognized typing phages Among those lysed at RTD 23 out of 214 group I strains (mainly type 80/81) and 66 out of 128 group III strains were susceptible, whereas 151 group II strains were resistant Among those typed at 1000 × RTD 10 out of 113 group III strains were lysed by 592, but none of the strains belonging to group I and II (188 and 101 strains, respectively)

71 previously non typable strains out of a total of 217 were classified as 'type 592' The resistance pattern of these, given in Table 2 shows that it is mainly the multiple resistant strains that are lysed

Temperate phages were isolated propagated, and designated as described by *Rosendal & Bulow* (1965) The method used for determination of their antigenic characters is that suggested by *Rountree* (1949)

The following strains were used as donor strains E 3253 (type 84/85/6557/592), E 2643 (84/85 E 3057 (85/592), E 3077 (6557/592) and E 3035 ("592 ') The phages were all propagated on E 3082 (84/85/6557/592) and they all belonged to serological group B The phages are designated by the number of their staphylococcal strain of origin followed by a prime sign

Lysogenization was carried out according to the method given by *Rosendal & Bulow* (1965) The lysogenized cultures are described in the usual way, *e g* 3252(3035') *is strain* 3252 *lysogenized* with phage 3035 *isolated from strain* 3035

Phage typing was done by the method of *Blair & Williams* (1961) with the phages 84 and 85 added to the set

Non typable strains and strains only typable at RTD × 1000 and classified as belonging to group III, the miscellaneous and mixed groups, or type 83A, were re tested with phages 84 and 85 together with the two experimental phages

Resistance to seven antibiotics (penicillin = P streptomycin = S tetracyclines = T, chloramphenicol = C, erythromycin = E, neomycin = N, methicillin = M) was determined by the technique described by *Jensen & Kiær* (1947) and *Drewsted & Erichsen* (1953) the material from 1968 was investigated by the methods previously used by *Bulow* (1968 a) Methicillin resistance has been determined by incubation at 30°C (*Hewitt et al* 1969)

Resistance to mercuric chloride was examined by the method given by *Moore* (1960) as described by *Jessen et al* (1963)

Production of lipase was demonstrated by use of a 'Tween 80/calcium agar (*Sierra* 1957)

RESULTS

The Complex and the Relationship between the Various Phage patterns

Table 3 gives a survey of the various phage patterns included in the complex It is seen that the strains are lysed by one or more of the phages 83A 84 85 6557 and 592' Strains lysed by phage 592 only represented 28 per cent, 26 per cent have the phage pattern 84/85/6557/592, 18 per cent

[1] Internationally designated as phage 89

TABLE 1 *Lytic Spectrum of Phage 592'*

Phage	Propagating strains														
	29	52	52A/79	80	2009	71	8719	42C	42E	47	53	54	73	75	77
592'	O	1	O	2	O	O	O	O	O	5	5	5	O	5	4

Strength of phage reaction
5 = maximum titre on homologous propagating strain
4 = $10^{-1} - 10^{-2}$ of titre on the propagating strain
3 = $10^{-3} - 10^{-4}$ of titre on the propagating strain
2 = $10^{-5} - 10^{-6}$ of titre on the propagating strain
1 = very weak lysis
O = no reaction

are lysed by 83A in addition, 6 per cent are lysed by 6557' only All other phage-patterns are represented by percentages lower than the above-mentioned

The relationship between strains lysed by phage 83A and those lysed by 84 and/or 85 has previously been demonstrated by *Jevons & Parker* (1964) *Bulow* (1968a, b) found that the majority of "type 6557" strains were lysed by the phages 84 and 85 as well, and that these strains had originated from type 83A

TABLE 2 *Antibiotic Sensitivity of 217 Non-Typable Strains Correlated with Sensitivity to Phage 592*

	Lysed by phage 592'	Resistant to phage 592'	Total
Sensitive to antibiotics or resistant to P and PS	2	138	140
Resistant to PST(C) PST(C)E PST(C)M, PST(C)EM	69	8	77
Total	71	146	217

P = penicillin
S = streptomycin
T = tetracyclines
E = erythromycin
M = methicillin
(C = strains are included whether they are resistant to chloramphenicol or not

22*

TABLE 3 *Phage patterns of Strains Included in the 83A, 84, 85, 6557, 592 Complex*

Phage pattern	Number of strains	Per cent of the total complex
83A/84/85/6557/592	149	18
other combinations with 83A included in the pattern	22	3
84/85/6557/592	216	26
84//85/592	16	2
84/85	9	1
84/6557/592	7	1
84/6557	8	1
84/592	14	2
84	7	1
85/6557/592	12	1
85/6557	6	1
85/592	24	3
85	9	1
6557/592	21	2
6557	54	6
592	236	28
other combinations = strains lysed at 1000 × RTD only	34	4
Total	844	

By analysis of 166 out of 251 strains described by *Bülow* (1968 a) as "type 6557", it was found that the provisional type did not represent an entity 6 per cent were lysed by 6557' solely, and 79 per cent were lysed by 84, 85 and 592' as well The remaining 15 per cent could be referred to other phage-patterns

"Type 592' has not previously been described From Table 4 it is seen that 592' lyses the majority of the strains of the complex, and the connexion of "type 592" with the other strains is furthermore confirmed by the finding that 182 out of a total of 222 strains lysed by various combinations of 84, 85 and 6557' at 1000 × RTD were lysed by 592' at RTD

TABLE 4 *Reactions with the Single Phages at RTD*

Typing phages	83A	84	85	6557'	592'
Number of strains lysed by the phage	168	430	443	483	703
Per cent of the total number of strains = 807	21	53	55	60	87

that of phage 84 nor 85, and strains lysed by 84, 85 and 6557' in various patterns seemed to differ with respect to antibiotic-resistance

In recent years a new wave of mostly non-typable methicillin resistant epidemically occurring strains has followed, and the Danish staphylococcal laboratory has found a new experimental phage (592')[1] useful *in typing* them, evidence suggests that these strains also belong to the complex which comprises strains lysed by the phages 83A, 84, 85 and 6557'

By re typing staphylococcal bacteraemia strains, preserved from the years 1962 68, with the phages 84, 85, 6557 and 592' it has been possible to follow the various phage-patterns back through the years and to register their occurrence and spread to the hospitals

Furthermore, it is the aim of the present report to give experimental evidence which would suggest relationship among strains of various phage-patterns included in the complex and to correlate the patterns with resistance to antibiotics

MATERIAL AND METHODS

Strains 2237 *Staphylococcus aureus* strains isolated from 2162 cases of septicaemia during the years 1962 1968 Part of the material has previously been made use of as described by *Jessen et al* (1969)

Experimental typing phages 6557 was isolated and described by *Bulow* (1968 a) 592 was isolated as a variant of phage 84 and propagated on a non typable strain (P S 592) *It belongs to serological group B The lytic spectrum given in Table 1 is* not identical with any of those of the phages 84, 85, D, 6557 (*Bulow* 1968 a)

Used at RTD only phage 592 was applied to various strains lysed by internationally recognized typing phages Among those lysed at RTD, 23 out of 214 group I strains (mainly type 80/81) and 66 out of 128 group III strains were susceptible whereas 151 group II strains were resistant Among those typed at 1000 × RTD 10 out of 113 group III strains were lysed by 592 but none of the strains belonging to group I and II (188 and 101 strains, respectively)

71 previously non typable strains out of a total of 217 were classified as 'type 592 The resistance pattern of these, given in Table 2 shows that it is mainly the multiple-resistant strains that are lysed

Temperate phages were isolated propagated and designated as described by *Rosendal & Bulow* (1965) The *method used for determination of their antigenic characters is that suggested by Rountree* (1949)

The following strains were used as donor strains E 3253 (type 84/85/6557/592), E 2643 (84 85 E 3057 (85/592), E 3077 (6557/592) and E 3035 ('592) The phages were all propagated on E 3082 (84/85/6557/592) and they all belonged to serological group B The phages are designated by the number of their staphylococcal strain of origin followed by a prime sign.

Lysogenization was carried out according to the method given by *Rosendal & Bulow* (1965) The *lysogenized cultures are described in the usual way e g* 3252(3035) *is strain 3252 lysogenized with phage 3035 isolated from strain 3035*

Phage typing was done by the method of *Blair & Williams* (1961) with the phages 84 and 85 added to the set

Non typable strains and strains only typable at RTD × 1000 and classified as belonging to group III, the miscellaneous and mixed groups, or type 83A, were re tested with phages 84 and 85 together with the two experimental phages

Resistance to seven antibiotics (penicillin = P *streptomycin* = S tetracyclines = T, chloramphenicol = C, erythromycin = E, *neomycin* = N methicillin = M) *was determined* by the technique described by *Jensen & Kiær* (1947) and *Drag sted & Erichsen* (1953) the material *from* 1968 was investigated by the methods *previously* used by *Bulow* (1968 a) Methicillin resistance *has* been determined by incubation at 30°C (*Hewitt et al* 1969)

Resistance to mercuric chloride was examined by the method given by *Moore* (1960) as described by *Jessen et al* (1963)

Production of lipase was demonstrated by using a 'Tween 80/calcium agar (*Sierra* 1957)

RESULTS

The Complex and the Relationship between the Various Phage-patterns

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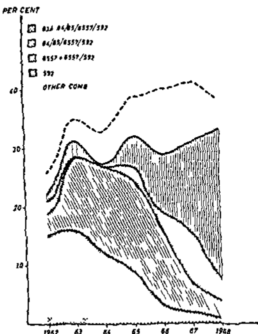


Fig 2 Occurrence of the various phage types

increasing Only one strain of this type was isolated in 1962 but in 1968 it was by far the most frequently isolated member, amounting to 62 per cent of the complex

Strains of phage types '6557 and '6557/592' show a peak in 1967, but their percentage contribution to the complex does not exceed 9

None of the remaining phage patterns making up a total of 20 per cent of the complex (Table 3) reaches the percentage level of the above mentioned types

Antibiotic Pattern Correlated with Phage Pattern

The correlation between antibiotic pattern and phage pattern can be seen from Fig 1 57 per cent of the strains within the complex resistant to PST but sensitive to the other antibiotics in question belong to type 83A/84/85/6557/592 and only a very few strains of this type are resistant to E and C One strain is methicillin resistant and none are neomycin resistant

The most frequent antibiotic pattern of type 84/85/6557/592 is PST(C)EN[1], and 84 per cent of strains with this antibiotic-resistance are type 84/85/6557/592 Resistance to E and N is found in 69 and 52 per cent, respectively, of this type, whereas only 9 per cent are M resistant

Seventy three per cent of the new "type 592" share the resistance-pattern PSTM, and 87 per cent of strains with this antibiotic-pattern belong to the new type It is noteworthy that "type 592' is never resistant to N

Seventy per cent of the strains lysed by phage 6557' either alone or in combination with 592' have the resistance pattern PST(C)EM, and only 4 per cent of the strains are N-resistant Among the strains with the resistance pattern PST(C)EM, 34 per cent are 'type 6557/(592)" and 31 per cent "type 592'

The number of the strains within the remaining typing patterns is too small to permit the designation of a typical resistance-pattern, but the pattern PSTM is frequent among phage type 85/592 Strains lysed by the phages 84 plus 85 are often resistant to E and N, and strains lysed by 84 solely are often resistant to E and M

Antibiotic Pattern Correlated with Time

As might be expected the various patterns of antibiotic resistance are subject to variations in time (Fig 3), following the distribution of the various phage types In 1962 83A/84/85/6557/592 was the most numerous type, and the most frequent resistance pattern was PST, in 1968 "type 592 is represented by the highest percentage, and 56 per cent of the strains are resistant to PSTM, which is the characteristic resistance of "type 592'

Spread of the Various Phage Types (Table 8)

Within the period 1962 68 cases of septicaemia caused by the 83A, 84, 85, 6557, 592 complex were diagnosed in 61 hospitals Very

[1] (C) in the resistance pattern means that strains are included in the group whether they are resistant to C or not

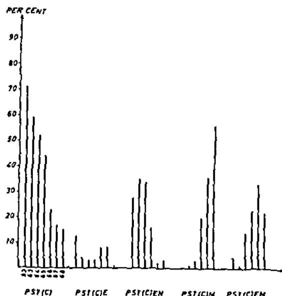


Fig 3 Occurrence of various antibiograms

few of these infections (8 per cent) could with certainty be said to have originated outside hospitals, as compared to 40 per cent of cases caused by other phage-types In view of this it may be permissible to consider these hospital originated septicaemia cases as evidence of the existence of a similar local hospital flora, if so, the figures from Table 8 give a picture of the distribution and spread of the various members of the complex in question over the country They also confirm the sequence of 83A/84/85/6557/592 and '592 as dominating epidemic types "6557/(592)' not found in 1964 caused an epidemic situation in one hospital in 1966

DISCUSSION

This report deals with a group of strains found to be closely related It still remains to be shown whether or not strains of the various phage patterns have a common ancestor and how the development has taken place

84/85/6557/592 seems to be a direct descendant of type 83A/84/85/6557/592 This is shown experimentally (*Jevons & Parker* 1964, *Bülow & Rosendal* 1964), and the sequence in time points in the same direction Furthermore, *Bulow* (1968a) found that 85 out of 87 strains the susceptibility of which to phage 83A was only partly blocked (typed at 1000 × RTD), could be re typed as 84/85/6557 But the phage patterns of the two remaining strains (84/6557 and 85/6557 respectively) show that other evolutionary events may take place

In the course of time an abbreviation of the typing pattern takes place This may be due to a series of lysogenization events but it is still not clear how many such events are needed in order to produce a type like for instance '592 Further investigations are difficult, as some of the prophages in question may be imperfect At any rate, many of the prophages blocking the lipase production in type 592 are imperfect The more distant relationship between the newest types and type 83A/84/85/6557/592 is also shown by the fact that TW+ variants of the former are not lysed by 83A as were the corresponding variants of type 84/85/6557/592

Other phage mediated mechanisms than lysogenization must be responsible for the changing resistance to antibiotics as non lysogenized variants never show a resistance pattern different from that of the parental strain According to *Ayliffe* (1970), resistance to neomycin seems — at least in strains not

TABLE 7 *Per Cent of Strains of the 83A, 84, 85 6557 592 Complex among Total Number of Bacteraemia Strains*

	1962	1963	1964	1965	1966	1967	1968	Total
Per cent of total number	26	35	33	39	40	41	40	38
Total number of strains	188	232	254	320	391	399	451	2237

TABLE 8 *Occurrence of Bacteraemia Cases in Hospitals*

Phage type	83A/84/85/6557/592				84/85/6557/592				6557/(592)				592			
Year	1962	1964	1966	1968	1962	1964	1966	1968	1962	1964	1966	1968	1962	1964	1966	1968
Number of cases per hospital	Number of hospitals															
1	5	9	6	4	3	13	11	5	4	0	6	6	1	1	12	15
2 or 3	5	2	3	0	2	5	7	4	0	0	2	3	0	0	4	6
4 or 5	1	2	0	0	0	0	2	0	0	0	0	0	0	0	0	5
6 or 7	1	0	0	0	0	2	0	1	0	0	0	1	0	0	0	2
8 or 9	0	1	0	0	0	0	2	0	0	0	1	0	0	0	2	2
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Total number of hospitals	12	14	9	4	5	20	22	10	4	0	9	10	1	1	18	32

6557/(592) indicates strains lysed by 6557 only and in combination with 592 *Cases with more than one phage type not included*

belonging to the 83A 84 85 6557 592 complex — to be a fairly unstable probably plasmid controlled character

The instability of the genetic determinants responsible for kanamycin/neomycin resistance seems according to our own investigations (to be published) and according to *Annear & Grubb* (1969) to be highly pronounced if the staphylococcal strains are also resistant to methicillin In Denmark the decrease of the number of neomycin resistant strains coincides with the emergence and increase of that of the methicillin resistant strains As this change cannot be correlated to restricted use of neomycin (*Bulow* 1971) as previously stated (*Jessen et al* 1969) the instability of the strains resistant to N+M offers a probable explanation

A rapid increase of strains resistant to methicillin has been observed in many countries (see *Parker & Hewitt* 1970) In 1969 *Dornbusch et al* found that methicillin resistance in strains of phage type 29 (1000 × RTD) was a transducable property and that the transductants—mostly non typable—were lysogenized

If these observations also hold true for strains belonging to the 83A complex it is more probable that transduction is the underlying mechanism of the shift of antibiotic resistance and that the transduced individuals become lysogenized at the same time owing to the simultaneous abundancy of phages In methicillin resistant strains the lysogenization causes a change of the phage pattern which is not seen among the neomycin resistant ones This is in concordance with the results obtained by *Ayliffe* (1970) who states that there is no difference in the phage patterns of the interdependent strains whether resistant to neomycin or not Related problems have recently been discussed in another report from this laboratory (*Jessen et al* 1969)

It is uncertain whether the evolution described here has a world wide application It all depends on local antibiotic policy and the properties of endemic bacterial strains and staphylococcal phages present in the environment (*Bulow* 1970)

The development has however taken place on a country wide scale But it has not been possible to determine whether the new types once originated have been introduced to other hospitals or wether they have originated independently in several hospitals owing to local conditions (*Bulow* 1968c) Furthermore it is seen that the newer types inside the hospitals have an advantage over the older ones of the complex in that they are able to replace them

Hospital environments are always subject

12290 and *S necrophorus* 3328 Institut Pasteur Strain N167 was cultivated in nutrient broth enriched with 0 5 per cent glucose, 0 3 per cent yeast extract and 0 1 per cent cysteine The pH was adjusted to 7 0 For cultivation of strains 12290 and 3328 the medium was supplemented with 0 0005 per cent haemin, 0 0001 per cent menadione and 4 per cent horse serum Bacteria in late logarithmic or early stationary phase of growth were harvested by centrifugation, washed three times in saline and dried with acetone

Extraction and purification methods were essentially the same as described earlier (5, 9) Briefly, acetone dried cells were extracted at room temperature with 45 per cent aqueous phenol (25), and the water phase fractionated by ultracentrifugation at 100 000 *x g* for 1 hr The washed pellet obtained by ultracentrifugation is referred to as LPS sed The designation LPS is used for the endotoxic lipopolysaccharide which was isolated from the supernatant fluid

Columns of Bio-Gel A–1 5 m 100–200 mesh agarose content 8 per cent (Bio Rad Laboratories Richmond Calif, USA) were used for gel filtration Columns for ion exchange chromatography were prepared from DEAE cellulose (DEAE SS Serva Heidelberg Germany) Digestion with ribonuclease (5 × cryst ex bovine pancreas) and deoxyribonuclease I (1 × cryst ex bovine pancreas stock DN C) both obtained from Sigma Chemical Company St Louis Miss, USA) took place in 0 1 M phosphate buffer, pH 7 0

Paper chromatography Acid hydrolysis was performed in sealed tubes at 100°C with 0 1 N H_2SO_4 for 10 min N H_2SO_4 for 4 or 16 hr or with 3 N HCl for 3 hr Salt free preparations were made from 10 min hydrolysates by neutralization with $Ba(OH)_2$ followed by centrifugation and evaporation of the supernatant to dryness *in vacuo* The 4 and 16 hr sulphuric acid hydrolysates were neutralized by a passage through a column of Dowex 1 in the formate form Acid was removed from the hydrochloric acid hydrolysates by evaporation in vacuo over NaOH pellets Sugars were separated by circular paper chromatography with *n* butanol pyridine water (6 4 3) or phenol water (4 1) and stained with silver nitrate or aniline hydrogen phthalate Amino sugars were also detected with the Elson Morgan reagent (18) The Warren thiobarbituric acid reagent (22) was used for detection of KDO

Chemical analyses Neutral sugars were measured by the Winzler orcinol method (26) with glucose galactose (1 1) as standard Hexosamine was estimated as glucosamineHCl by the method of *Rondle & Morgan* (20) Samples were hydrolysed at 100°C with 3 N HCl for 4 hr The thiobarbituric acid method (23) was used for detection and determination of KDO Samples were hydrolysed in 0 02 N H_2SO_4 for 20 min at 100°C (17)

Heptose was sought as described by *Duche* (4) Fatty acid esters were determined as tripalmitin by the hydroxamic acid method (21) Nitrogen was determined by the micro-Kjeldahl method (11) Samples were digested for 4 hr The Folin Ciocalteau phenol method (14) was used for estimation of protein Bovine serum albumin served as standard

Double diffusion in agar was performed as described (6) Antisera were prepared against whole microbes (6)

Local Shwartzman reactions were produced in 6 months old albino rabbits (7) Skin lesions were recorded immediately before and 18 hr after the provocative injection

EXPERIMENTS AND RESULTS

Preparation of LPS

The pellet obtained after ultracentrifugation of the water phase following phenol water extraction of all three strains (LPS sed) contained considerable amounts of glycogen Thus the LPS sed preparations contained up to 90 per cent carbohydrate and the prevalent sugars in acid hydrolysates were glucose and an oligo saccharide, which also was found in acid hydrolysates of glycogen Attempts to remove this contaminant from the LPS sed preparations failed

LPS was therefore prepared from the supernatant fluid after ultracentrifugation by a procedure recently described (9) By means of this procedure serologically active material in the supernatant with the same specificity on agar precipitation as LPS sed is separated from serologically inactive UV-absorbing substances The purification methods include treatment of the freeze-dried supernatant with ribonuclease and deoxyribonuclease gel filtration on Bio-Gel A 1.5 m and ion exchange chromatography with DEAE cellulose

In a representative experiment 300 mg freeze dried supernatant fluid 12290 was suspended in 60 ml 0 1 M phosphate buffer pH 7 0 containing 6 mg ribonuclease and 3 mg deoxyribonuclease After incubation in a 37°C water bath for 1 hr storage at 4°C overnight and reduction of the volume by evaporation at 10°C to approximately 15 ml the digest was applied to a 2 5 × 50 cm

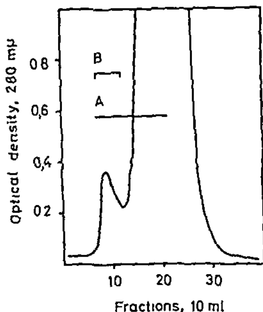


Fig 1 Gel filtration of 300 mg lyophilized enzyme treated supernatant fluid 12290 on Bio Gel A 1 5 m A Serologically active material (agar precipitation) B Blue dextran (Separate run)

column of Bio Gel A 1 5 m The agarose gel had been stabilized with 0 1 M tris (hydroxy methyl) amino methane buffer pH 7 8 containing 0 001 M EDTA and 0 02 per cent sodium azide Elution was performed with the same buffer at a flow rate of 10 ml/hr (Fig 1) Serologically active material as measured by agar precipitation against anti serum *12290* appeared in the void volume and in fractions up to no 21 Fractions 7–14 were collected The subsequent serologically active and contaminated fractions (fractions 15 21) were subjected to a new run on the same column of Bio Gel A 1 5 m after dialysis against tap water overnight and reduction of the volume by evaporation Most of the precipitating material now appeared in the void volume well separated from the bulk of UV absorbing material These fractions and fractions 7 14 from the first run were pooled and dialyzed overnight against tap water After reduction of the volume by evaporation to approximately 15 ml the material was applied to a 1 5 × 30 cm column of DEAE cellulose, previously equilibrated with 0 02 M phosphate buffer pH 7 4 Elution took place overnight with the same buffer at a flow rate of 10 ml/hr A linear NaCl gradient in the buffer was then used for elution of the serologically active LPS, which appeared in the eluate between 0 04 and 0 5 M NaCl The yield of freeze dried dialyzed LPS 12290 was 39 mg

The elution patterns of supernatant 3328 on Bio Gel A 1 5 m and DEAE cellulose were the same as described for supernatant 12290 and LPS 3328 was obtained in similar yields *ii* about 10 per cent of freeze dried supernatant Supernatant N167 showed the same pattern of elution on Bio Gel A 1 5 m Serologically active material invariably appeared with the first peak of UV absorbing material from DEAE cellulose columns (cfr Fig 2), and had to be discarded However, the bulk of serologically active material was eluted between 0 04 to 0 7 M NaCl The yield of lyophilized LPS N167 from freeze dried supernatant varied around 20 per cent in different experiments

Chemical Composition of LPS

The sugars identified by chromatographic analysis of acid hydrolysates of LPS from the three *S necrophorus* strains are listed in Table 1 KDO and heptose were also demon

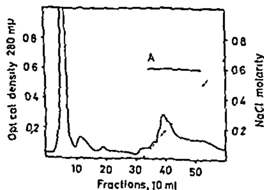


Fig 2 Chromatography of serologically active material from Bio-Gel A 1 5 m on DEAE-cellulose at pH 7 4 A Serologically active material (LPS 12290)

12290 and *S necrophorus* 3328 Institut Pasteur Strain N167 was cultivated in nutrient broth enriched with 0 5 per cent glucose 0 3 per cent yeast extract and 0 1 per cent cysteine The pH was adjusted to 7 0 For cultivation of strains 12290 and 3328 the medium was supplemented with 0 0005 per cent haemin 0 0001 per cent menadione and 4 per cent horse serum Bacteria in late logarithmic or early stationary phase of growth were harvested by centrifugation washed three times in saline and dried with acetone

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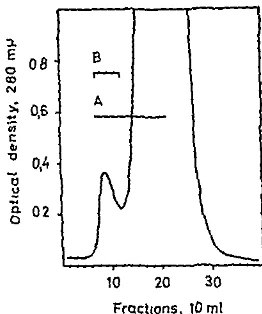


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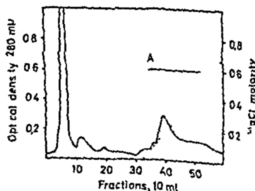


Fig 2 Chromatography of serologically active material from Bio-Gel A-1 5 m on DEAE-cellulose at pH 7 4 A Serologically active material (LPS 12290)

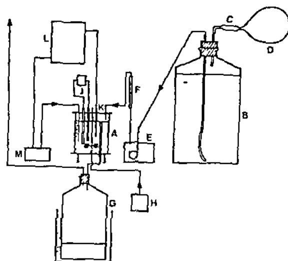


Fig 1 Cultivation equipment

A Culture vessel
B Medium reservoir
C Gas filter
D Rubber balloon (N_2 + CO)
E Hose pressure pump
F Medium flow meter
G Cooled collecting flask
H Gas tube (N_2 + CO_2)
J Thermo-regulation
K pH electrode
L pH titrator
M Alkali pump

varied and was measured with a flow meter (F) The culture volume was kept constant by an internally placed overflow tube the culture overflow being collected in a flask immersed in an ice bath (G) The rubber tubing connecting the reservoir for fresh medium with the fermentor was made as short as possible to minimize the risk of air diffusion through the wall of tubing This tubing was connected to the reservoir by a syringe needle inserted through a rubber membrane in the stopper of the reservoir Thus the reservoir could easily be changed aseptically by disconnecting the needle and inserting it through the membrane of a new reservoir The reservoir was connected by a sterile gas filter (C) to a rubber balloon (D) filled with a gas mixture of 95 per cent (v/v) N_2 and 5 per cent (v/v) CO_2 to avoid a negative pressure in the reservoir as it was emptied In order to maintain an anaerobic atmosphere in the fermentor during growth the same gas mixture was bubbled from a gas tube (H) via a sterile gas filter through a tube situated under the impeller The flow rate of the gas was 1 5 l per hour The outgoing gas was exhausted through the same tube as the overflowing culture *The rate of the stirrer was 600 rpm* Temperature control (J) was effected by means of a controlling thermometer placed in a pocket in the fermentor, an electronic relay and a 40 watt cartridge heater (Biotec, Stockholm Sweden) An automatic titrator (L) was used for the control of pH (Type TTT1, Radiometer Copenhagen, Denmark) The titrator was equipped with a MNR1 magnetic relay connected to a hose pressure pump (M) For neutralization 5 M NaOH was used The pH of the culture was checked every day by an independent measurement on a culture sample The culture vessel, the reservoir the rubber and glass tubing connecting them and the sampling flasks were sterilized by autoclaving and assembled aseptically The pH electrode (K) (Radiometer GK 2021 c) was sterilized in 1 per cent (v/v) β propiolactone (Fluka AG, Buchs Switzerland) before being inserted aseptically into the fermentor The culture was inoculated with 100 ml of a fresh static culture grown in thioglycolate medium U S P (Oxoid) This inoculum gave an initial density of approximately 10 mg of cells per l Immediately after inoculation a sample was taken and pH measured Adjustment of the pH titrator was then effected if necessary, and the control function started The culture was allowed to grow batchwise for seven hours before starting the continuous addition of fresh medium Samples were collected at 16 and 40 hours after each change of the growth conditions and used for determination of cell dry weight, proteolytic activity glucose, and volatile fatty acids Dry weights were measured on 10 ml samples as described earlier (14)

Chemical analyses Glucose was analysed enzymatically (Glox AB Kabi Stockholm Sweden) using a Technicon Automatic Analyzer

The culture supernatant was analysed for volatile fatty acids after precipitating the protein with $Zn(OH)_2$ according to Neish (12) A Varian 1400 gas chromatograph was used The column filling was acid washed Chromosorb W (60/80 mesh) coated with 31 2 per cent PEGA (2) The carrier gas (N_2) flow rate was 80 ml/min and the temperature programming 100° 150° (4°/min)

Enzyme analysis Proteolytic activity was determined according to *Kunitz* (11 A 2 per cent casein solution (Merck) heat denatured at 100°C for 10 minutes and adjusted to pH 7 4 was used as substrate Of this substrate 2 ml was mixed with 1 ml 0 1 M phosphate buffer (pH 7 4) 0 5 ml 0 01 M $CaCl_2$ and 0 5 ml 0 01 M cystein 0 2 ml of the sample was added and the mixture incubated at 37°C The reaction was stopped after 30 minutes by adding 3 ml 10 per cent perchloric acid After centrifugation the absorbance of the supernatant was read at 280 nm against a reaction blank for each sample One enzyme unit was defined as the amount of enzyme which gives an absorbance of 1 0 at 280nm of the supernatant after incubation for 30 min followed by precipitation with perchloric acid

Enzyme preparation Samples taken from the culture were centrifuged at 3000 × g for 30 minutes at 4° C The cells and the supernatant were kept frozen at −20°C until required for analyses Two methods were used for disintegration of the cells freeze pressing and sonic disruption Disintegration of frozen cells was achieved by freeze pressing as described by *Edebo* (3) The disintegrated cells were suspended in 0 01 M phosphate buffer (pH 7 0) and centrifuged at 20 000 × g for 10 minutes at 4°C The supernatant was then analysed for proteolytic activity For sonic disintegration the frozen cells were thawed by suspending them in 0 01 M phosphate buffer (pH 7 0) at 0°C to a density of approximately 2 mg cell dry weight per ml The suspension was then disintegrated in a M S E sonic disintegrator at 20 kC per sec in 15 ml batches for 1 minute at 0°C, after which the suspension was centrifuged in the same way as after freeze pressing

RESULTS

The culture vessel was made as simple as possible without impairing safety of operation In its present form it is reliable and easy to handle and has been used repeatedly for continuous cultures of 3–4 weeks' duration The maximum working capacity is 850 ml This volume of culture was used, since we required relatively large amounts of cell material The influence of different growth conditions on the cell yield and the formation of proteolytic activity was studied Each figure represents one experiment All experiments were repeated twice with good reproducibility As optimal conditions for high cell yields were not always the same as those for maximum production of proteolytic activity, the results are presented separately

The main acidic end products formed were acetic acid and n butyric acid These acids were formed in equimolar amounts Approximately 1 5 moles of acid were formed per mole of glucose consumed as calculated by the amount of sodium hydroxide required to maintain a constant pH in the cultures

Influence of dilution rate on cell yield *Sph necrophorus* was grown in the basal medium at pH 7 0 and 37°C at different dilution rates (Fig 2) The cultures were started at a low dilution rate which was increased stepwise every second day In some experiments, the dilution rate was again decreased back to the initial value Dilution rates below 0 17 hr⁻¹ were accompanied by extensive lysis of the culture Washout occurred at dilution rates above 0 40 hr⁻¹ In these experiments the glucose concentration was 10 g/lit and constituted the growthlimiting factor The cell yield was constant at dilution

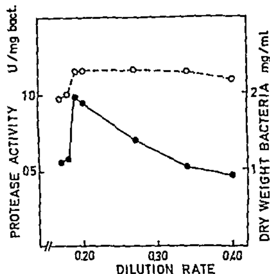


Fig 2 Bacterial dry weight (O------O) and protease activity (●——●) at different dilution rates in continuous culture of *Sph necrophorus*

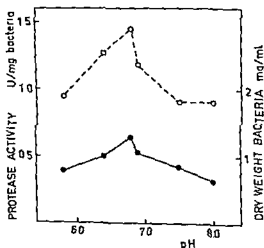


Fig 3 Bacterial dry weight (O------O) and protease activity (●——●) at different pH in continuous culture of *Sph necrophorus*

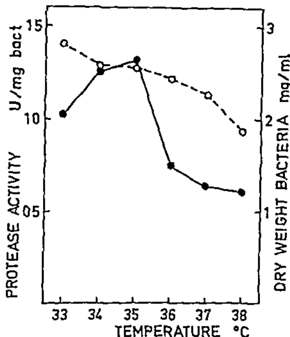


Fig 4 Bacterial dry weight (○ – – ○) and protease activity (●——●) at different temperatures in continuous culture of *Sph necrophorus*

rates between 0 19 hr ¹ and 0 40 hr ¹ at a dry weight of 2 2–2 3 g/lit

Influence of pH on cell yield The influence of pH on the cell yield was tested in the basal medium at 37°C and a dilution rate of 0 22 hr ¹ The cultures were started at pH 7 0 The pH was first decreased step wise and then increased back to 7 0 and higher values (Fig 3) Steady state growth was obtained at pH levels between 5 8 and 8 0 At pH 8 5 there was extensive lysis and slimy aggregates accumulated in the culture The maximum cell yield 2 9 g/lit was obtained at pH 6 8 The cell yield was the same for each pH level whether the pH was increased stepwise or it was decreased back to the same values Glucose was the growth limiting factor

Influence of temperature on cell yield The influence of temperature on cell yields was tested in the range 33°–38°C in the basal medium at pH 6 8 and a dilution rate of 0 22 hr ¹ (Fig 4) In these experiments the culture was started at 37°C The temperature was lowered 1°C at a time and then raised in the same way Steady state was reached for each temperature tested, the cell yield in a culture at a particular temperature was the same whether the temperature was being lowered or it was being raised again The highest cell yield, 2 8 g/lit, was obtained at 33°C, but the difference between this and the yield at 36°C was only slight

Influence of medium composition on cell yield The composition of the basal medium was then varied in an effort to obtain a still higher yield Cell yield was proportional to the glucose concentration up to 15 g/lit (Fig 5), indicating that up to this level the growth limiting factor was glucose At 15 g/lit, the cell yield was 3 5 g/lit Only *slightly larger yields were obtained at higher* glucose concentrations indicating that growth above this level is limited by some other as yet unidentified, component A change in the tryptone concentration from 15 to 25 g/lit had no significant effect on cell yield (Fig 6) The cell yield rose from 1 7 to 2 2 g/l

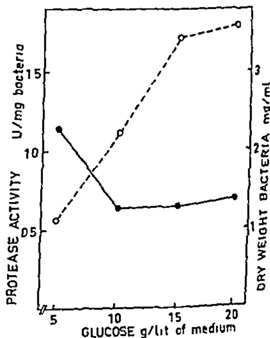


Fig 5 Bacterial dry weight (○ – – ○) and protease activity (●——●) at different glucose concentrations in continuous culture of *Sph necrophorus*

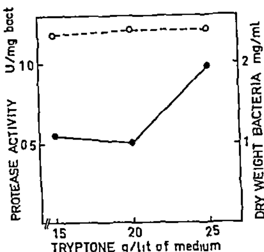


Fig 6 Bacterial dry weight (O---- O) and protease activity (●——●) at different tryptone concentrations in continuous culture of *Sph necrophorus*

when the yeast extract concentration was increased from 1 to 5 g/l A further increase did not stimulate total growth Growth in continuous cultures could be maintained without yeast extract if $MgSO_4$ (10^{-3} M), a solution of trace metals (4) and 1 ml of B vitamin mixture (Bevitotal, Astra, Sweden), were added instead The cell yield however, was only 1 4 g/lit under optimal conditions for growth in this medium, nor could it be improved by increasing the concentrations of trace metals or vitamins It was thus found that the optimal conditions for a high cell yield in the basal medium were dilution rates between 0 19 hr^{-1} and 0 40 hr^{-1} pH 6 8 and a temperature of 33°–36°C Furthermore, the optimal glucose concentration was 15 g/lit and cell yield was not affected by adding more tryptone and yeast extract

Proteolytic activity Proteolytic activity was located almost entirely to the bacterial cells less than 2 per cent of the total activity being found in the culture fluid Samples collected from the continuous culture and kept at 37° for 30 min autolyzed to more than 50 per cent Proteolytic activity was also very low in the supernatant after centrifugation of the autolyzed culture After sonic treatment of the bacteria, approximately 95 per cent of the total proteolytic activity was located in the soluble fraction, the remaining 5 per cent being found in the pellet After differential centrifugation according to *Salton* (13) and enzyme analysis of the resulting fractions, only trace activities remained in the washed cell wall fraction and less than 2 per cent of the total activity in the cell debris No significant difference in proteolytic activity was observed after freeze pressing, compared with sonic disintegration, and the latter technique was used for routine work, since it is more rapid and since the amount of cells processed in one operation is sufficient for accurate measurement of enzyme activity It was essential not to keep the cells frozen for more than 2 days before analysis In cells kept at —20°C, proteolytic activity fell by approximately 30 per cent in one week The subsequent decline was somewhat slower, the activity after 45 days' storage amounting to approximately 40 per cent of the initial value

The growth rate of the cells markedly influenced proteolytic activity (Fig 2) Maximum activity was observed at a dilution rate of 0 19 hr^{-1} Dilution rates above 0 20 hr^{-1} were accompanied by a marked fall in activity, while at rates below 0 19 hr^{-1} lysis in the culture increased and the proteolytic activity fell sharply At a dilution rate of 0 17 hr^{-1} the cell yield was approximately 12 per cent lower than at 0 19 hr^{-1} and the proteolytic activity was 40 per cent lower

The proteolytic activities of cultures grown at different pH are shown in Fig 3 The pH optimum appears to be the same for cell growth and proteolytic activity Formation of proteolytic activity Formation of proteolytic activity during continuous cultivation at different temperatures is shown in Fig 4 The marked reduction at temperatures above 35°C accounts for the low protease activity obtained in early experiments which were performed at 37°C

An increase in the tryptone concentration from 15 to 20 g/lit did not stimulate the proteolytic activity but at 25 g/lit there was

TABLE 1 *Yields of Bacteria and Protease in Continuous Culture at Different Concentrations of Glucose or Sucrose Dilution Rate 0 2 h⁻¹, Temp 35°, pH 6 8*

	Glucose			Sucrose		
	5 g	10 g	15 g	5 g	10 g	15 g
Cell yield (mg/ml, d w)	1 15	2 25	3 50	1 30	2 20	2 58
Protease (U/mg cells)	1 15	0 64	0 65	0 66	0 71	0 70
Protease (U/ml culture)	1 32	1 44	2 28	0 86	1 56	1 81

a considerable rise (Fig 6) Alterations of the concentration of yeast extract in the medium did not significantly affect the formation of protease In the basal medium, the growth-limiting factor was glucose Variations in the glucose concentration from 5 to 20 g/lit showed that cells grown in glucose concentrations above 5 g/lit had a markedly lower formation of proteolytic activity (Fig 5)

To avoid the inhibitory effect by glucose on the proteolytic activity *Sph necrophorus* was also grown in a medium in which sucrose was used instead of glucose (Table 1) Sucrose concentrations up to 20 g/lit were tested and had no inhibitory effect on the protease formation The proteolytic activity achieved was, however, lower than that to be achieved with glucose at the optimal concentration No glucose could be detected in the culture fluid in these experiments The cell yields, however, were lower (2 6 g/lit for 15 g/lit sucrose) than with glucose The optimal growth conditions for the formation of proteolytic activity were as follows a growth rate equivalent to a dilution rate of 0 19 hr⁻¹, pH 6 8 and a temperature of 35°C In continuous culture the peak activity was obtained in a medium with a high concentration of tryptone, and glucose as the main energy source, at an input concentration of 5 g/lit Under these conditions no glucose could be detected in the culture fluid

DISCUSSION

The continuous cultures were operated for up to 40 days The duration of individual runs was limited by accumulative bacterial growth on the pH-electrode, which made it impossible to control the pH adequately However, for most practical purposes a cultivation time of up to 30 days is sufficient With the range of dilution rates applied in the cultures of *Sphaerophorus*, a minimum of 14 steady state levels could be investigated during each run Furthermore, having discontinued one culture, only two days were needed to have a new culture in continuous operation in the same unit

It was not necessary to remove traces of oxygen from the commercial gas mixture indicating that *Sph necrophorus* is less sensitive in this respect than the bacteria grown by *Hobson & Summers* (8) As mentioned earlier (9, 10), oxygen diffuses through the walls of rubber tubes Therefore, the use of rubber tubing was limited to short pieces connecting glass tubes and in the hose pumps Eh was not measured in the cultures though it would be possible to fit the vessel with a further electrode for this purpose A comparatively large culture volume was used so that the samples would be large enough to permit isolation and purification of cell-bound proteolytic enzymes

The cell yield in continuous cultures grown with 10 g glucose per lit was higher (2 2 2 9 g/lit) than the yields obtained in batch cultures (1 8 g/lit) (14 The media were the same except for the addition of buffer salts to the continuous culture medium The batch cultures were run at 37°C and pH 7.0 in the continuous cultures it was found that the optimum for cell yield was 35°C and pH 6 8 There seemed to be less bacteriolysis under these conditions the appearance of slimy aggregates which is an indication of lysis often occurred in batch cultures but was not observed in continuous cultures run under optimal conditions A quantitative estimation of lysis was not performed because the accuracy of such determinations is very

low when applied to growing cultures in complex media The lower degree of lysis however, was not solely responsible for the higher cell yields in the continuous cultures Glucose was the limiting factor, and its initial concentration was the same both in batch and continuous culture In the continuous culture however, the concentration of glucose during steady state growth was very low and consequently the use of other energy sources may be less inhibited Calculations on the cell yields indicate that additional energy sources were used by *Sphaerophorus* in these cultures (14)

Approximately 1 5 moles of acid were formed for each mole of glucose consumed at the dilution rate optimal for cell yield This is less than that formed in batch cultures indicating that in continuous cultures the use of energy sources other than glucose may influence the consumption of alkali

When the dilution rate was increased from 0 19 hr^{-1} to 0 40 hr^{-1} and with it the glucose concentration in the culture the amount of acids formed per mole of glucose increased from 1 5 to 2 1, indicating that glucose had a repressive effect on the use of other energy sources

The proteolytic activity was found to be cell bound However only traces of proteolytic activity were observed in the supernatant of cultures which were subject to extensive lysis a conceivable explanation being that inhibitory substances were released from spontaneously lysing cultures or that the culture supernatant contained inhibitory substances When the bacteria were disrupted by sonic disintegration however the same inhibition was not obtained

The cultures tended to lyse at dilution rates below 0 19 hr^{-1} Below 0 17 hr^{-1} this tendency was so pronounced that it ruled out steady state growth The proteolytic activity in cells grown at dilution rates below 0 19 hr^{-1} was much lower than in cells grown at 0 19 hr^{-1} The reason for this observation could be that bacteria on the verge of lysis released proteolytic enzymes to the culture fluid where it could not be detected The proteolytic activity declined as the concentration of glucose in the medium rose, possibly suggesting an inhibition of protease formation by glucose (Table 1) However, the cell yields indicated that the culture was glucose-limited up to a glucose concentration of 15 g/lit, so that a low glucose concentration was to be expected in the culture In spite of this protease activity decreased when the glucose concentration was increased from 5 to 10 g/lit Other mechanisms may lie behind the decreasing protease activity at high glucose concentrations, the concentration of tryptone and other constituents of the medium necessary for the formation of proteolytic enzymes may not be sufficient at the higher cell yields obtained when the glucose concentration was increased above 5 g/lit This hypothesis is supported by the observation that a tryptone concentration of 25 g/lit had a pronounced stimulatory effect on the formation of proteolytic activity (Fig 6) While the proteolytic activity in this culture was enhanced by an increased tryptone concentration this was not the case for the cell yield since the culture was still glucose limited No inhibition of enzyme formation was observed at high concentrations of sucrose The proteolytic activity remained practically the same in sucrose concentrations ranging from 5 to 15 g/lit, whereas the cell yield increased with the sucrose concentration (Table 1) Moreover the yield of proteolytic activity per cell mass never reached the values obtained at low concentrations of glucose

Some of the results in this study are rather difficult to explain possibly because they may depend on the presence of more than one proteolytic enzyme However further experiments including the isolation and characterization of the proteolytic activity are presently under way

This work was supported by the *Swedish Medical Research Council* (Project No B69 16X-2185-04)

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Acta path microbiol scand Section B 79, 399–405 1971

INFLUENCE OF CULTIVATION CONDITIONS ON THE PRODUCTION OF EXTRACELLULAR PROTEINS BY *STAPHYLOCOCCUS AUREUS*

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Three strains of *Staphylococcus aureus*, selected for their capacity of producing various enzymes and toxins, were cultivated on membrane-covered solid medium and in liquid media using shake flasks and stirred aerated fermentors A medium was used in which the pH changes did not exceed 0 4 units during the period of active growth Yeast extract was required as a medium component for high yields of cells and extracellular proteins Cultivation in liquid medium was superior to membrane covered solid medium for the production of both cells and extracellular proteins Maximal bacterial yields were obtained in aerated cultures Although the activities of some of the exoenzymes calculated per cell weight were lower in aerated cultures as compared with non aerated, cultivation in the stirred aerated fermentor was found to be the best method for the production of large amounts of extracellular proteins from *Staphylococcus aureus*

Several cultivation techniques have been employed in different laboratories for the production of extracellular proteins from *Staphylococcus aureus* The methods used most extensively are cultivation on solid media covered with a semipermeable membrane (5 10 14 28) in shake flasks (3, 9, 21 26) and in stirred fermentors (1, 19, 27) The present study was performed in order to find a cultivation method, by which maximal amounts of exotoxins and exoenzymes could be obtained for purification and characterization

The technique using membrane-covered agar media has been shown to give very good yields of a number of exoproteins This method however has several disadvantages as compared to cultivation in liquid culture The pH cannot be controlled adequately, aeration efficiency cannot be estimated during growth and scaling up is difficult The stirred, aerated fermentor, on the other hand, offers all possibilities for control of culture conditions and scaling up, and may be used for continuous culture Since the basic conditions are widely different between cultivation in liquid and on solid media, experiments were carried out to evaluate the particular characteristics of each method for the production of staphylococcal exoproteins The choice of the optimal cultivation method could not be based on results given in the literature, because of differences in the assay procedures used by different workers, rendering a comparison between their yields impossible

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the ability to produce different toxins and enzymes, e g for α-toxin (22), nuclease (20), hyaluronate lyase (16), and lipase (13) Three strains were used in the present study strain V8 which has been reported to give high yields of leucocidin (8), strain Wood 46 which has been selected for high production of α toxin (7), and strain M18 which gives high yields of hyaluronate lyase (16, 17, 18)

MATERIALS AND METHODS

Bacterial Strains

The *Staphylococcus aureus* strain V8 was obtained from Prof *G P Gladstone*, University of Oxford, England, *strain Wood 46 (NCTC 10345)* from Dr *E Kjems*, Statens Seruminstitut, Copenhagen, Denmark, and strain M18 from Prof *J Schmidt*, University of Leipzig, Germany

Culture Medium

The medium was based on the Woodin modification of the "CCY medium" (27) described by *Gladstone & van Heyningen* (8)

1	Casein hydrolysate (Oxoid)	40	g
	Yeast extract (Difco)	10	g
	Sodium β-glycerophosphate	20	g
	Sodium lactate (50 per cent)	10	ml
	Na_2HPO_4 $2H_2O$	1	g
	KH_2PO_4	0 4	g
	$(NH_4)_2SO_4$	1	g
	dl-tryptophane	80	mg
	l-cystine	100	mg
	Dist water	1000	ml
2	Vitamin stock solution		
	Thiamine	20	mg
	Nicotinic acid	40	mg
	Dist water	100	ml
3	Trace elements stock solution		
	$MgSO_4 7H_2O$	0 2	g
	$MnSO_4 4H_2O$	0 1	g
	$FeSO_4 7H_2O$	0 06	g
	Citric acid	0 06	g
	Dist water	*100*	*ml*

The bulk of the medium was sterilized at 120° The pH of the medium was 7 0 Polypropylene glycol *P-2000 (Dow Chemicals Midland, Mich)* [illegible] before sterilization [illegible] ins [illegible] solution of trace elements also was sterilized separately After sterilization 10 ml of each stock solution were added for each litre of the final medium

Cultivation techniques Precultures were inoculated with the growth from a fresh nutrient agar slant They were grown for 18 h at 37° in Trypticase Soy Broth (BBL, Cockeysville, Md, USA) *in Erlenmeyer flasks on a rotary shaker*

Cultivation on dialysis membrane was performed on 19 cm plates on CCY-medium solidified by addition of 1 5 per cent Difco agar The membrane used was *Cuprophan 150 PT* (Bemberg Wuppertal, Germany) The cultures were incubated at 37° for 18 h The membrane was then transferred to a new Petri dish and carefully washed using in total 50 ml of *0 1 M* phosphate buffer (pH *7 0*

A culture volume of 100 ml was shaken *in 1-l* Erlenmeyer flasks with or without indentations The former flasks had 4 indentations approximately 15 mm in depth The flasks were shaken on a *rotary shaker at 120 r p m and having a rotation* radius of 25 mm, in a constant temperature room at 37° Aeration efficiency, as determined by the sulphite oxidation method (6) was approximately *150 mmoles O_2 per l and h in the indented flasks* and less than 5 in the ordinary flasks

Cultivation in stirred fermentors was performed in a Biotec FL 103 unit (Biotec, Stockholm Sweden) The culture volume was 2 5 l *Agitation* was performed by a vaned disc stirring at a speed of 800 r p m No baffles were used The air flow rate was 0 5 l per min The aeration efficiency as estimated by the sulphite oxidation *method (6)* was approximately 100 mmoles O_2 per l and h under these conditions The pH was maintained constant by means of an automatic titrator (TTT1 Radiometer, Copenhagen, *Denmark*), equipped *with a MNR 1 magnetic* relay connected to a hose [illegible] electrode

Determination of Toxins and Enzymes

α toxin The *procedure* used for the determination of α toxin has been reported earlier (22)

Hyaluronate lyase Hyaluronate lyase activity was determined according to *Vesterberg* (24) The *definition of the enzyme* unit has been changed however, to conform with international standards It is defined as the amount of enzyme which gives an increase in absorbance at 230 *nm* of 1 0 *in one min*

Lipase Lipolytic activity was estimated according to *San Clemente & Vadhera* (15) as modified by *Vesterberg* (unpublished data) Tributyrine was used *as the* substrate in an emulsion of 10 per cent (w/v) 1 0 ml of this emulsion was used in the reaction mixture which also contained 5 5 ml 0 15 M sodium chloride *and* 1 0 ml 0 1 M *calcium chloride* 0 1 ml of a suitable dilution of the sample was added The pH titrator was set at pH 8 0 and kept at this pH by automatic addition of 0 025 M sodium *hydroxide to the* reaction mix

ture which was kept at 37° in the titration vessel The reaction was recorded for 5 min and linearity for this time was always obtained One unit of enzymatic activity is defined as the amount of enzyme which liberates 1 μeq of acid in one min

Phosphatase Acid phosphatase activity was determined using p nitrophenylphosphate (Sigma Chemical Co, St Louis, Missouri, USA) 0 5 ml of the substrate (4 mg/ml), was mixed with 0 5 ml succinic acid sodium hydroxide buffer at pH 5 3 and 0 05 ml 0 1 M magnesium sulphate 0 02 ml of the sample was added and the reaction mixture was incubated for 30 min at 37° The reaction was terminated by adding 5 ml 0 04 M sodium hydroxide The developed colour was assayed at 410 nm using a Beckman B spectrophotometer (4) One unit of enzymatic activity is defined as the amount of enzyme, which liberates 1 μmole of p nitrophenol in 60 min

Protease Proteolytic activity was determined according to *Kunitz* (12), using heat denatured casein as the substrate 2 ml of a 2 per cent casein solution (Merck), heat denatured at 100° for 10 min and adjusted to pH 7 4 was mixed with 2 ml of a 0 05 M phosphate buffer at pH 7 4, containing 1mM calcium chloride and 1mM cystein 0 2 ml of the sample was added and the reaction mixture was incubated for 30 min at 37° The reaction was terminated by adding 3 ml of 10 per cent perchloric acid After centrifugation the absorbance of the supernatant was read at 280 nm against a reaction blank for each sample One unit of enzymatic activity is defined as the amount of enzyme which gives an absorbance of 1 0 at 280 nm of the supernatant after precipitation with perchloric acid after an incubation time of 30 min

Staphylokinase Staphylokinase activity was determined according to *Vesterberg et al* (25)

Bacteriolytic activity This was determined according to *Wadstrom & Hisatsune* (23), using a suspension of *M lysodeikticus* cells as the substrate (11)

RESULTS

Influence of medium composition The influence of medium components on yields of cells and extracellular proteins was first studied using strain V8 grown in shake flasks The defined medium given by *Gladstone* (7) was first tested A cell yield of 1 5 g/l was obtained but the yields of toxins and enzymes were very low The medium described by *Gladstone & van Heyningen* (8) was then modified to include sufficient quantities of organic nitrogen sources and vitamins to meet the demands of the increased cell yields obtained in aerated cultures Since acid hydrolysed casein was used, tryptophane and cystine were added to the medium Since the yeast extract was heat sterilized at pH 7 0 an additional amount of the vitamins thiamin and nicotinic acid was also included in the medium From Table 1 it can be seen that an increase in the concentration of casein hydrolysate and the addition of thiamine and nicotinic acid resulted in higher cell yields A series of batch cultivations using the stirred fermentor was then performed in order to test the influence of medium composition, pH and aeration conditions on the yields of bacterial cells and extracellular proteins The results of these studies will be reported in a subsequent paper (2) The cell yields obtained at pH 6 8 were 8 0 9 0 g d w per l for strain V8, 7 5 8 0 g/l for Wood 46, and 8 5 9 5 g/l for M18, using medium 6 of Table 1 The three strains were also grown in a medium without yeast extract (medium 4, Table 1) The activities of all enzymes and leucocidin were very low in these cultures The yields of α toxin were, however, the same as in medium 6

TABLE 1 *Bacterial Yields at 24 h of* Staphylococcus aureus *Strain V8, in Shake Flasks Using Different Concentrations of Casein Hydrolysate, and the Influence of Yeast Extract and Added Vitamins**

	Casein hydrol (g/l)	Yeast extr (g/l)	Thiamin Nicot ac	Cell yield dry weight (g/l)
1	20	0	—	1 5
2	20	0	+	3 5
3	20	10	+	6 5
4	40	0	+	4 9
5	40	10	—	7 0
6	40	10	+	8 2

* All medium components not listed were added in the concentrations used in the modified CCY-medium

Comparison between Different Cultivation Techniques

In Table 2 yields of bacteria and extracellular proteins obtained by different cultivation techniques are given The yields of

TABLE 2 *Yields of Bacteria and Extracellular Proteins by Different Cultivation Techniques The Enzyme Activities Are Given in Units per mg of Cells (Dry Weight)*

Strain V8	Time (h)	dry weight (mg/ml)	Protease	Phosphatase	Staphylokinase
Membrane plates	24	1 60*	2 3	4 8	0 2
Shake flask without indent	8	2 5	2 8	18	2 8
	24	2 5	1 0		
Shake flask with indent	8	6 2	2 5†	7 3	5 8
	24	8 1	0 2		
Stirred fermentor with active aeration	8	8 5	2 5	10	5 3
	24	10 1	<0 1	11	1 9

Strain M18	Time (h)	Dry weight (mg/ml)	Bacteriolytic activity	Lipase	Phosphatase	Hyaluronate lyase
Membrane plates	24	1 52*	0	30	2 6	2 5
Shake flasks without indent	8	2 0	0	68	15	5 2
	24	1 9	0			
Shake flasks with indent	8	6 9	2 2	79	5 2	4 2
	24	6 6	0 8			
Stirred fermentor with active aeration	8	8 7	2 9	60	4 1	3 2
	24	10 2	0 8	76	4 5	2 1

Strain Wood 46	Time (h)	Dry weight (mg/ml)	α toxin
Membrane plates	24	0 78*	500
Shake flasks without indent	8	2 8	360
Shake flasks with indent	8	7 5	270
Stirred fermentor with active aeration	8	5 5	1800
	24	6 9	1450

* mg/cm²
† 6 h

enzymes and toxins are represented as units produced per mg of bacteria This was done in order to enable a comparison of yields from cultures in liquid and on solid media The different strains varied in their ability to produce the various enzymes The enzymes listed in Table 2 are those which were best produced by the particular strain Since the bacterial yields in liquid cultures varied considerably depending on the aeration efficiency, the enzyme yield per unit volume is also given (Table 3) The yields represented for the liquid cultures and the membrane cultures are given as the 8 h and 24 h value respectively The calculated yield from 10 membrane plates representing an area of 0 25 m, are also given in Table 3 This number of plates was chosen as a basis for comparison, since the time and labour involved appeared to correspond to a batch culture in a stirred fermentor

Since some of the enzyme activities decreased markedly during the stationary phase in liquid media it was essential to analyse several samples from each culture The peak activities were always formed after 6 8 h of cultivation which represented the end of the logarithmic phase

For all enzymes cultivation in liquid media was superior to membrane plates (Tables 2 and 3) Strain V8 was the best producer of protease phosphatase and staphylokinase whereas strain M18 formed the highest amounts of bacteriolytic enzymes lipase and

TABLE 3 *Comparison of Extracellular Protein Yields from* Staphylococcus aureus *Produced by Different Cultivation Technique**

Strain V8	Protease	Phosphatase	Staphylokinase	Dry weight (g/l)
Membrane plates	9,200	19,200	800	4 0†
Shake flasks without indent	7,000	45 000	7 000	2 5
Shake flasks with indent	15 500	45 300	36,000	6 2
Stirred fermentor	21,300	85,000	45,100	8 5

Strain M18	Bacteriolytic activity	Lipase	Phosphatase	*Hyaluronate* lyase	Dry weight (g/l)
Membrane plate	0	114,000	9,900	9 500	3 8†
Shake flasks without indent	0	136,000	30,000	10,400	2 0
Shake flasks with indent	15,200	545 000	35 900	29 000	6 9
Stirred fermentor	25 200	522,000	35 700	27,800	8 7

Strain Wood 46	α toxin	Dry weight (g/l)
Membrane plates	1×10^6	2 0†
Shake flasks without indent	1×10^6	2 6
Shake flasks *with indent*	2×10^6	7 5
Stirred fermentor with active aeration	10×10^6	5 5

* The values given for the membrane plates represent the total yield of enzyme units per 10 plates (0 25 m²) The time of cultivation was 24 h *For the liquid cultures the total yield from 1 l is given The time of cultivation was 6–8 h*
† g/10 plates

hyaluronate lyase Strain Wood 46 was superior to the other *strains in the production* of α toxin

As can be seen in Table 2, the enzyme yields per mg of cells displayed a variable response to the aeration efficiency Increased yields at high *aeration were obtained* of staphylokinase, bacteriolytic enzymes and α toxin, decreased yields of phosphatase and hyaluronate lyase, whereas no significant difference in yields was recorded for protease and lipase (Table 2) However, as the cell yields were much higher in cultures with active aeration, the enzyme yields per culture volume were highest in the *stirred fermentor* or in the indented shake flasks (Table 3) The *enzyme yields obtained by these latter* two methods of cultivation were generally of the same order of magnitude

The 24 h values of enzymatic activities were low in the liquid cultures for protease, *staphylokinase, and for bacteriolytic activity* This has been shown to depend on an instability of these enzymes The protease activity of strain V8 depends on at least three different enzymes, one of which is unstable under the conditions of cultivation (unpublished data) The proteolytic activity was more rapidly inactivated in vigorously stirred and aerated cultures than, e g in the cultures grown in shake flasks without *indentations* This pronounced loss of activity was not *observed for α-toxin*, phosphatase, lipase, and hyaluronate lyase

DISCUSSION

Cultivation in liquid media offers great advantages with respect to environmental control and scaling up Since the cell yields of staphylococci were increased considerably if efficient aeration *was applied, cultivation in* stirred fermentors should be favourable *Hallander (10) showed*, however, that growth on solid media, covered with cellophane, gave improved yields of a number of staphylococcal exoproteins as compared with shake flask cultures Since it could not be

excluded that the enzyme yield *per* cell weight could be higher on the solid medium than in liquid culture, a comparison was made between different culture methods generally used for the laboratory scale production of staphylococcal cells and exoproteins. Due to differences in the assay procedures, it was not possible to compare the yields of toxins and enzymes reported by *Hallander* (10) with the yields obtained by other workers and by ourselves.

It is difficult to estimate the time and labour involved in the different cultivation methods. If small amounts of toxins and enzymes are to be produced, the membrane plate method is convenient, for a number of exoproteins it gives a high concentration in a small volume. For the purification and characterization of the exoproteins the amounts required are generally so large that the surface area of solid medium needed will be too big to be practical. Even for protease where the yield per cell weight is almost as high on membrane plates as in the stirred fermentor, an area of 6 m² will be required to correspond to a 10 l batch culture.

Since the pH of the cultures did not change more than 0.2-0.4 units during the first 8 h of cultivation, shake flask cultures could be used for small scale production of the exoproteins. In these the effect of aeration could be estimated. Shake flasks provided with indentations give a moderate efficiency of aeration, whereas ordinary non-indented flasks have a very low aeration efficiency. The yields of bacterial cells were always considerably improved at the higher aeration. The yields of exoproteins however displayed different responses.

Several factors appear to influence the enzyme yield. The inactivation of the exoproteins can be more rapid in the agitated and aerated culture, and enzyme inhibitors may be formed during growth or released from autolysed cells. Therefore, no conclusions can be drawn about the influence of aeration on the rate of enzyme formation until these factors have been explored. Furthermore, several activities measured consist of more than one enzyme and the different components may have different stability under the conditions of cultivation.

In the stirred fermentor, the cell yields were higher than in the indented shake flasks although the aeration efficiency as measured by the sulphite oxidation method was lower than in the shake flasks. In the shake flasks, however, the oxygen concentration in the gas over the culture decreased gradually during growth and the aeration efficiency became lower. The stirred fermentor, on the other hand, was provided with fresh air continuously, and therefore offers a more reproducible method of cultivation. The aeration conditions in the fermentors could be increased, but at the high cell densities obtained foaming was intense and became a major technical problem. It was found that the enzyme yields either did not increase in proportion to the cell yields if extensive foaming occurred or they could even decrease.

An advantage of the membrane plate method is that only dialysable medium constituents are recovered in the harvest. However, the casein hydrolysate contains very small amounts of non-dialysable matter and diffusate of yeast extract is commercially available. In the stirred fermentor cultures, more than half of the non-dialysable substances originated from autolysed bacteria and therefore it was not considered necessary to dialyse the yeast extract. A completely dialysable medium would be desirable if the products of cultivation are to be used for immunization.

The skilful technical assistance provided by Mrs *Ingegerd Friberg*, Miss *Berit Lindholm* and Mrs *Ingrid Mollegård* is gratefully acknowledged. This work has been supported by the *Swedish Board for Technical Development*, Grant No 68-335-U and by *Emil och Wera Cornell Stiftelse*.

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28

TABLE 3 *The Enzymatic Activities per g of Cells (Dry Weight) in the Culture Supernatants from Strain M18 at the Point of the Growth Curve Where the Bacterial Dry Weight Was 6 3 g/l at Different pH At pH 5 5 and 9 0 This Cell Concentration Was Not Obtained and the Values at the End of the Growth Period Are Given*

pH of cultivation	DNase U/mg d w	Hyalase U/mg d w	Lipase U/mg d w	Phosphatase U/mg d w
5 5	0	0 19	15 6	0 35
6 0	24	0 57	19 8	1 00
6 5	30	1 62	51 5	1 20
7 0	102	2 75	59 5	2 95
7 5	83	2 61	59 5	2 15
8 0	59	0 62	51 5	1 20
8 5	51	0 19	35 6	0 70
9 0	43	0	0	0 10

TABLE 4 *The Enzymatic Activities per g of Cells (Dry Weight) in the Culture Supernatants from Strain Wood 46 at the Point on the Growth Curve Where the Bacterial Dry Weight Was 6 3 g/l at Different pH At pH 5 5 This Cell Concentration Was Not Obtained and the Value at the End of the Growth Period Is Given*

pH of cultivation	α toxin U/mg d w	DNase U/mg d w	Lipase U/mg d w	Phosphatase U/mg d w	Protease U/mg d w
5 5	300	0	0	1 0	0 28
6 0	1580	5 2	11 4	1 0	0 41
6 5	1580	17 5	23 8	1 6	0 50
7 0	2540	21 4	13 5	2 1	0 69
7 5	1270	21 4	9 5	2 8	0 55
8 0	630	15 0	3 9	2 2	0 43
8 5	475	9 5	1 6	0 8	0 12
9 0	0	4 7	0	0 3	0 08

were obtained in cultures of strain Wood 46 Strain M18 displayed very low activities The maximum yield was obtained at pH 6 5 7 0 The enzyme yield per g of cells had the same pH maximum The increase in protease activity in the culture supernatant stopped

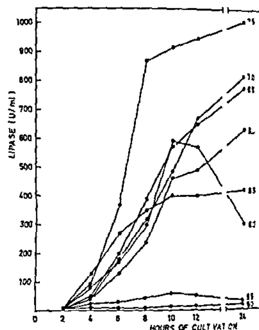


Fig 3 Production of lipase during 24 h of growth of *Staphylococcus aureus* strain M18 at different pH

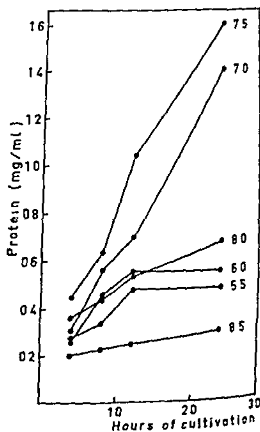


Fig 4 Total extracellular protein in culture supernatants *Staphylococcus aureus* strain M18 grown at different pH

at the end of the growth period at pH 7 0 8 5 During the stationary phase there was always a 25 50 per cent decrease in activity at pH above 6 5

Total extracellular protein The total protein content in culture supernatants from strain M18 was twice that obtained from strain Wood 46 when cultivated at pH 6 5-8 0 At more acid or alkaline pH there was no difference The maximum was at pH 7 0 7 5 for both strains The rate of increase of extracellular protein during cultivation of Wood 46 declined after 12 h and only a slight increase was seen during the stationary phase Strain M18, however, released considerable amounts of protein after the cessation of bacterial growth when cultivated at pH 7 0 and 7 5 (Fig 4)

DISCUSSION

pH is known to have an influence on the production of extracellular proteins from *Staphylococcus aureus* *Tirunarayanan* (17) cultivated two strains without aeration in nutrient broth and a casein hydrolysate medium and found that the production of lipase and phosphatase had two maxima lipase at pH 6 5 and 8 25 and phosphatase at pH 5 5 and a second at pH 7 25 None of these findings could be reproduced in our investigation which might be explained by differences in aeration conditions and in the composition of the nutrient medium A comparison between the yields obtained at optimal conditions by *Tirunarayanan* and in this study could be made only for hyaluronate lyase and lipase The conversion factor for hyaluronate lyase was determined by control experiments using both methods on the same enzyme preparation Due to differences in the assay methods the yields of the other toxins and enzymes could not be compared For hyaluronate lyase the highest yield obtained by *Tirunarayanan* (17) was approximately 0 1 U/ml This value is in the same order of magnitude as that obtained with our strain Wood 46 This shows that the choice of strain is of great importance, since the maximum yield from strain M18 was 28 5 U/ml

For lipase, a similar assay method was used in both studies (2, 17) Although different substrains of Wood 46 was used the ability to produce lipase should be similar The yield obtained by *Tirunarayanan* was approximately 1 3 U/ml, whereas the yield obtained in this investigation was 150 U/ml from Wood 46 and 1010 U/ml from strain M18

Release of cell bound exoproteins has been found at high ionic strength of the surrounding medium (5) The differences in salt concentration in cultures run at different pH, caused by the neutralizing agents added, were very small and could not have influenced the yields significantly Furthermore, the optimal exoprotein yields were achieved at pH 6 5 7 5, where the smallest amounts of neutralizing agents were required

The present study shows that the production of extracellular toxins and enzymes from staphylococci has an optimal pH range between 6 5 and 7 5 The production of total extracellular proteins per unit cell mass decreases rapidly with increasing pH At pH 9 0 the growth yield was still high but only DNase was produced in significant amounts Also at pH 6 0 the bacterial growth was good, but the production of all enzymes was very low *Hendricks* & *Altenbern* (8) found that the pH interfered with *α* toxin synthesis of *S aureus* on the intracellular level When the culture became more alkaline than pH 7 4, toxin synthesis declined rapidly even though the growth proceeded at an unaltered rate It does not seem very probable that the synthesis of exoproteins should be more sensitive to variations in the pH than the synthesis of cellular proteins unless the former takes place outside the cytoplasmic membrane, where the pH is likely to be close to the pH of the surrounding medium (11) *Holzman et al* (9) have recently provided some evidence to the effect that the ribosomes responsible for the synthesis of penicillinase in *S aureus* were located on the outside of the cell membrane

The stability of the exoproteins under the cultivation conditions is of great importance for the final yields *Desmanzeaud et al* (7) showed that the production of protease from *Micrococcus caseolyticus* was maximal in cultures with an initial pH 7 0 The production ceased when the pH became more acid than pH 6 0 and the protease was inactivated By keeping the pH constant they could increase the yield and no inactivation was seen The same findings were made in our experiments The optimum for production of protease was at pH 6 5 and no inactivation was observed at this pH (Table 2b) The lower yields at higher pH seems to be due to instability of the enzyme which is continuously inactivated during the cultivation At pH below 6 5 no inactivation of the protease was seen The low yield per mg of bacterial dry weight at pH 5 5 and 6 0 is probably due to decreased synthesis or liberation

The inactivation of hyaluronate lyase was faster at pH values below 6 5 and above 8 0 and started before the end of the growth period Preliminary experiments showed that hyalase was stable for several hours at 37° between pH 5 5 and 8 5 The inactivation during cultivation must partly be due to factors other than the pH, such as denaturation in the foam oxidation, or liberation of intracellular inhibitors from lysed cells (16)

Alpha toxin, DNase, lipase and phosphatase were not inactivated during the cultivation The decreased production at acid and alkaline pH must be explained by inhibited synthesis transport or release *Coles & Gross* (5) have shown that the extent of liberation of penicillinase form *S aureus* caused by phosphate and some other anions was dependent on the pH Maximum release was obtained at pH 7 0 8 0 An inhibition of the transport could also inhibit the synthesis by means of a feed back mechanism The increase of some activities after the end of the growth period (Table 1b and 2b) is either due to release of preformed enzymes and toxin or to a continued production at a lower rate There was always a slight increase in the bacterial dry weight between 12 and 24 h of cultivation Bacterial lysis during the cultivation cannot be excluded The significant increase in total extracellular protein during the late part of cultivation is probably caused by lysis (Fig 4)

The skilful technical assistance provided by Mrs *Mona Olsson* and Miss *Berit Lindholm* is gratefully acknowledged This work has been supported by the *Swedish Board for Technical Development* (Grant No *68 335 f*) and by *Emil och Vera Cornells stiftelse*

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A CONTINUOUS PROCESS FOR THE PRODUCTION OF EXTRACELLULAR PROTEINS BY *STAPHYLOCOCCUS AUREUS*

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A continuous process has been developed for the production of staphylococcal enzymes and toxins In order to obtain sufficient quantities of these extracellular proteins, 40 l volumes of culture were produced by a short-term continuous process in a stirred fermentor with a working volume of 2 2 l Three strains of *Staphylococcus aureus*, selected for high capacity in producing various enzymes and toxins, were used Yeast extract was required as a medium component for optimal yields of the extracellular proteins Active aeration pH control and prevention of foaming were also essential for high yields In a series of cultivations the following growth conditions were selected dilution rate 0 5 h^{-1}, pH 6 8, air flow 0 5 l per min, and impeller speed 800 rpm Each run was terminated after approximately 36 h. Constant levels were obtained after 4 h of the continuous run for bacterial dry weight α toxin, leucocidin, DNase, phosphatase, protease, and staphylokinase Lipase activity increased during 20 h before constant levels were obtained Hyaluronate lyase decreased during the whole period of each experiment The activity levels of phosphatase, protease, and staphylokinase were approximately the same as those in batch cultures grown under the same conditions (2) The yields of α toxin and DNase were approximately half of those obtained in batch cultures The average yield of hyaluronate lyase per 40 l of culture supernatant from continuous culture was lower, and the average yield of lipase was higher than that obtained by the corresponding batch process

It is often difficult to produce extracellular enzymes and toxins from bacteria in amounts sufficient for purification and characterization The production of staphylococcal leucocidin in 80 l batches of strain V8 was reported by *Woodin* (17) The same strain was used for the production of a nuclease in 100-150 l scale by *Anfinsen et al* (1) Pronounced variations between different strains of *Staphylococcus aureus* in their ability to produce different toxins and enzymes have been observed, e g for α toxin (16), nuclease (15), hyaluronate lyase (14), and lipase (10 Three strains were used in the present study strain V8 which has been reported to give high yields of leucocidin (17) strain Wood 46 which has been selected for high production of α-toxin (16 and strain MC which gives high yields of hyaluronate lyase (13, 14)

In the present investigation a short term continuous process was adopted for the production of a range of staphylococcal extracellular proteins The advantage of using

Received 22.x.70

continuous process for this purpose was that the varying demands for obtaining sufficient amounts of the specific toxins or enzymes could be met by employing different times of cultivation, using the same size of fermentor for all cultivations A unit with a working volume of 2 2 l was used It was convenient in operation and provided good aeration conditions an essential factor when high yields of the extracellular proteins are to be obtained

MATERIALS AND METHODS

Bacterial Strains

The *Staphylococcus aureus* strain V8 was obtained from Prof *G P Gladstone* University of Oxford England strain Wood 46 (NCTC 10345) from Prof *A W Bernheimer* New York University New York N Y and strain M18 from Prof *J Schmidt*, University of Leipzig, Germany

Culture Media

Precultures were grown in Trypticase Soy Broth (BBL Cockeysville Md, USA) For the continuous cultures a modification of the CCY medium was used (2)

Precultures

The growth from a fresh nutrient agar slant was used to inoculate the precultures The latter were grown for 18 h at 37° in 800 ml Trypticase Soy Broth (TSB) on a rotary shaker in a 5 l Erlenmeyer flask The preculture was centrifuged in sterile tubes at 3500 × g for 30 min and the cells were suspended in 40 ml of the medium This suspension was used as inoculum for the stirred fermentor The bacterial yield in the TSB was 1 1 5 g dry weight per l Thus the inoculation density was 0 4–0 5 g per l

Cultivation Equipment

A stirred fermentor with a working volume of 2 2 l was used (Biotec FL 103 Biotec Bromma Sweden) Fresh medium was pumped from a 50 l spherical Pyrex glass flask by a hose pressure pump The flow rate was measured with a flow meter The culture volume was maintained constant by an internally placed overflow tube Air was supplied through a tube situated under the impeller and the flow rate was measured with a flow meter The pH was maintained constant by means of an automatic titrator (TTT 1 Radiometer Copenhagen Denmark) equipped with a MNR 1 magnetic relay connected to a hose pressure pump A combined glass calomel electrode was used (GK 2021C) Temperature control was effected by the use of a controlling thermometer placed in a stainless steel pocket immersed in the culture, a transistorized relay, and a Biotic cartridge heater of 40 watts' capacity The temperature fluctuations of the cultures did not exceed 0 2° and the pH was controlled with an accuracy of ± 0 05 pH units.

The culture overflow was conveyed through a spiral cooler kept at 0° with the aid of circulating ethylene glycol from a cooling unit The cooled culture was kept at 0° in a 4 l receiving flask in an ice bath The outgoing air was exhausted through the same tube as the culture overflow It passed through a glass wool filter maintained at approximately 60° with the aid of a heating tape (Electrothermal London England) to prevent condensation of moisture

Operation and Growth Conditions

The cultivation equipment was connected as shown in Fig 1 The pH electrode was sterilized in 3 per cent (v/v) β propiolactone (Fluka AG Buchs, Switzerland) prior to insertion in the fermentor Immediately after inoculation a sample was taken and the pH measured Adjustment of the pH titrator was then effected if necessary, and the control function was started Sodium hydroxide (2M) or lactic acid (40 per cent) was used for titration The culture was agitated by a vaned disc stirrer at a speed of 800 rpm Since excessive foaming occurred when baffles were used they were removed from the vessels used in these cultivations The air flow rate was 0 5 l per min The temperature was regulated at 37° and pH at 6 8 The aeration efficiency under these conditions was approximately 100 mmoles O per l and h as measured by the sulphite oxidation method (5)

Polypropylene glycol P 2000 was used as an antifoam Since the amount of antifoam in the incoming nutrient medium proved to be insufficient for effective foam control sterile undiluted polyglycol was added dropwise to the culture A 5 l flask was placed in the exit air line for safety reasons, in order to trap foam which occasionally overflowed from the receiving flask

The culture was initiated batchwise, the bacterial density being checked by wet weight determinations When a wet weight of approximately 30 g per l corresponding to 6 g dry weight, was achieved the continuous feed was started with a dilution rate of 0 5 h^{-1} Dissolved oxygen was measured with the electrode described by *Borkowski & Johnson* (4) The concentration of dissolved oxygen in the culture decreased during batch growth and was zero at a bacterial density of 4 5 g per l (dry weight) It then remained at zero during the continuous run

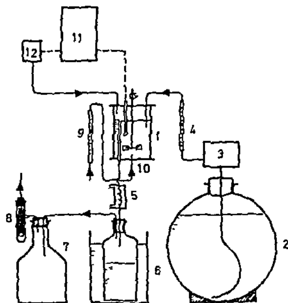


Fig 1 Cultivation equipment

1 Culture vessel
2 Medium reservoir
3 Hose pressure pump
4 Medium flow meter
5 Cooling spiral for outgoing culture
6 Cooled collecting flask
7 Foam trap
8 Air filter
9 Air flow meter
10 Air inlet
11 pH titrator
12 Alkali pump

Harvesting

The cooled culture l quid collected in the receiving flask was harvested every third hour by centrifugation in an International PR-2 centrifuge at 4° (3500 × g for 30 min) The supernatant was decanted and to prevent bacterial growth n butanol was added to give a concentration of 1 per cent (v/v) It was observed that lysis of bacteria occurred in the cooled culture liquid collected during a continuous run Bacteria stored overnight at +4° lysed up to 30 *per cent* as estimated by dry weight determinations Since the supernatants were used as starting material for the purification of extracellular proteins the harvested culture was centrifuged every third hour to minimize contamination with intracellular material

Determination of Toxins and En ymes

Leucocidin was estimated by the method of *Gladstone & van Heyningen* (9) using human polymorphonuclear leucocytes Twofold dilutions of culture supernatants were made in Hank s balanced salt solution Since no ant le con l n was available, the end point was determ ned as the highest dilution where a leucoc dal ef ect could be observed The reciprocal of th s ra e represents the leucocidin activity The accuracy of the test was estimated to ± one dilution step The procedures used for the determination of o her toxins and enzymes have been descr bed earl er (2 3)

RESULTS

Culture conditions The choice of medium, dilution rate, aeration conditions and pH for the continuous cultivation was based on experiences from batch cultures (2, 3) Optimal enzyme yields were obtained when the fermentors were aerated and stirred to give an aeration efficiency corresponding to about 100 mmoles O per l and h as determined by the sulphite oxidation method When the aeration efficiency was increased by increasing the impeller speed or the air flow rate foam formation became extensive and enzyme yields decreased

In batch cultures operated at optimum aeration efficiency, the dissolved oxygen concentration decreased to zero at a bacterial density of 4 5 g per l (dry weight) From this point, a linear increase in the bacterial density was obtained corresponding to about 2 5 g dry weight increase per h This period of oxygen limited growth was maintained for 2 3 h The rate of formation of extracell lar proteins was high In the continuous cultures the same aeration conditions were appl ed It could be shown that oxygen was the limiting factor, since the concentration of dissolved oxygen was zero during the continuous process The electrode described by *Borkowski & Johnson* was used (4) for measuring dissolved oxygen Increasing the aeration efficiency by mixing the incoming air with oxygen or increasing impeller speed or air flow rate resulted in a temporary increase in dissolved oxygen followed by an increased cell density and again a decrease in dissolved oxygen to zero The growth was thus oxygen limited and the bacterial density decreased with increasing dilution rate The dilution rate chosen 0 5 h⁻¹ gave a cell density of 6-7

g per l (dry weight) and was close to the maximum productivity value for cell production under oxygen limited conditions At higher cell densities the formation of foam increased greatly This made the addition of large amounts of a chemical foam breaker necessary, which has been shown to substantially decrease the oxygen transfer rate (7) Furthermore, the yields of toxins and enzymes were much lower in cultures with extensive formation of foam

It has been shown earlier (3) that the yield of exoproteins was low in defined media as compared to media containing casein hydrolysate and yeast extract Growth limitation by a nutrient factor in the medium was therefore not easily applied Since good production of the extracellular proteins was obtained at pH 6 8, and the pH during steady state growth generally remained at this value without external control in the medium used, this pH level was chosen for the production cultivations

Exoprotein production Since the production of extracellular proteins was the purpose of the cultivations, the facilities for handling large volumes of liquid determined the time for the continuous run A volume of 40 l was regularly required, and the majority of the cultures were consequently operated for approximately 36 h The three strains studied were grown 5 times each, and the activities of the different toxins and enzymes and the bacterial dry weights were determined (Table 1) The capacity of the strains to produce various toxins and enzymes differed greatly Strain V8 gave high yields of leucocidin, phosphatase protease, and staphylokinase, whereas strain M18 was the best producer of DNase hyaluronate lyase, and lipase Strain Wood 46 was used only for the production of α toxin In comparison with batch cultures the same enzyme concentrations were obtained in continuous cultures of strain V8 for phosphatase, protease, and staphylokinase The yields of DNase (strain M18) and α toxin (strain Wood 46) were approximately half those obtained in batch cultures (2)

TABLE 1 *Ranges of Activities of Different Toxins and Enzymes and of the Bacterial Dry Weight in Continuous Cultures of* Staphylococcus aureus *Strains V8, M18, and Wood 46* * *The values represent the ranges of activities obtained in five cultures of each strain*

	V8	M18	Wood 46
α toxin	100–200	100–400	4000–8000
Leucocidin	1000–2000	<100	–
DNase	<50	200–400	150–200
Hyaluronate lyase	<4	12–30†	<4
Lipase	200–800§	200–1500§	100–250§
Phosphatase	40–50	30–40	–
Protease	10–15	<1	–
Staphylokinase	30–40	30–40	–
Bacterial dry weight (g/l)	6 0–6 5	6 0–6 5	6 5–7 5

* Activities of toxins and enzymes are given in units/ml

† Decreased successively during the continuous run

§ Increased successively during the continuous run

The continuous feed was started when the bacterial density was 5-6 g per l (dry weight) Steady state levels of bacterial dry weight toxins and enzymes were apparently achieved within 4 h after the start of the continuous feed Two notable exceptions were experienced, however (Fig 2) In the cultures of strain M18, hyaluronate lyase decreased from a maximum value of approximately 30

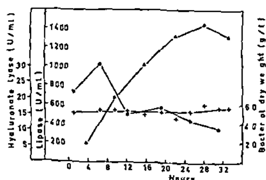


Fig 2 Bacterial dry weight hyaluronate lyase and lipase activities in a continuous culture of *Staphylococcus aureus* M18 The continuous feed was started at time zero

+——+ Bacterial dry weight
●——● Hyaluronate lyase activity
▲——▲ Lipase activity

U/ml at 3-4 h after the start of the continuous run to below 10 U/ml by the end of the cultivation period. Lipolytic activity, on the other hand, increased from an initial level of 200-300 U/ml to 1200-1500 in approximately 20 h (Fig. 2), then it appeared to remain at this level. An increase in the lipolytic activity during the continuous run was observed also in the case of the strains V8 and Wood 46 although the maximum activities obtained were lower than those obtained with strain M18 (Table 1).

It was found that very small amounts of lactic acid were required to maintain a constant pH during the continuous growth. The pH of the incoming medium was 7.0. The pH of the culture in the steady state was 6.8-6.9 without pH control. Therefore, pH control was omitted whenever an accurate pH level was not required. Measurement of pH was, however, always made in the cultures.

In preliminary studies, using batch cultivation, a pH stabilizing effect was observed if the incoming air were mixed with carbon dioxide (25 per cent) and improved cell yields were recorded. When automatic pH control was applied no effect of the carbon dioxide on the yields of cells, toxins, or enzymes was obtained. Therefore, no carbon dioxide enrichment of the incoming air was used in the present investigation.

The formation of foam was the main technical problem in the cultures. Maximal aeration efficiency could not be maintained, since the air flow and impeller speed had to be kept at moderate values to minimize foaming which was greatest during the late logarithmic phase of the batch culture by which the continuous run was started. After the commencement of the continuous feed foam formation was greatly reduced but the addition of substantial quantities of a chemical antifoam was always required. Silicone emulsions and polypropylene glycol (PPG) appeared to be equally effective as antifoam agents, but the enzyme yields were lower when silicone was used. Therefore PPG was used at a concentration of 0.05 ml per l medium before sterilization. The concentration of PPG in the medium entering the culture, however, was lower because of its limited solubility in water. Further addition of PPG directly to the growth vessel was therefore required in most cultures but the amounts of PPG needed varied for the different strains, M18 requiring 0.5 ml each half hour and Wood 46 0.5 ml per h. In the case of the strain V8, additional antifoam was generally not required.

DISCUSSION

Since the yields for most of the exoproteins were similar in continuous and batch culture a continuous process for large scale production should be considered. Small culture vessels, with capacities of 1-10 l can be used. The control of foaming, aeration conditions and pH is easier in this scale and it is also to be perferred for the handling of pathogenic micro-organisms. Harvesting, which is a serious problem with pathogens on a large scale, is much simpler in a continuous process.

For the start of a continuous culture, batch growth was allowed to proceed to a bacterial density where the rate of enzyme formation was maximal. When the continuous feed was started with a dilution rate of 0.5 h^{-1} the toxins and enzymes continued to increase for 2-4 h. Then the activities were constant except for hyaluronate lyase and lipase. The hyaluronate lyase activity decreased gradually during the whole 36 h run whereas lipase increased during the first 20 h and then remained constant (Fig. 2). *Rogers* (12) observed an initial increase in the rate of hyaluronate lyase synthesis during the first hours of continuous growth of staphylococci. This increase corresponds however to the initial increase in all the enzymatic activities obtained also in our experiments. The slow decrease in the rate of hyaluronate lyase synthesis and the increase in the rate of lipase synthesis which followed must be ascribed to the specific environmental conditions in the continuous culture. *Coulter* (6) observed that mutants with low α-toxin producing

capacity replaced the original high producing strain in continuous cultures of strain Wood 46 Only 5 per cent of the original titre was obtained after a cultivation period of 2 weeks

A certain variation in the levels of α toxin leucocidin DNase phosphatase, and staphylokinase weren often experienced during a continuous run The bacterial density on the other hand was generally constant Inactivation of the extracellular proteins in the foam was probably one the reasons for this variability, since the addition of the chemical foam breaker was performed intermittently

Another disturbance of the steady state also occurred if the foam control did not function properly Since an internal overflow was used the culture volume decreased if a layer of foam of increasing depth was permitted When the foam was then broken by addition of the antifoam some time lapsed until the liquid level had risen sufficiently for the culture overflow to function once again Therefore automatic addition of the antifoam agent and an external levelling device should offer great advantages in this respect

Lysis of the bacteria was probably the main cause of the foaming The quantitative estimation of lysis in a growing culture in a complex medium is difficult The amount of intracellular proteins released into the culture liquid probably varied between cultures of different strains since the tendency to produce foam varied For this reason the possibility must be considered that some of the enzymes or part of an enzymatic activity may not have been truly extracellular (11) Therefore washed bacteria were disintegrated by freezepressing (8) and the supernatants after disruption were tested for enzymatic activities The activities found were very low for all enzymes except lipase and staphylokinase The significance of the fact that part of these activities were cellbound under the conditions of cultivation used is presently under investigation

Our thanks are due to Miss *Berit Lindholm* for taking great interest in the development of the cultivation techniques Skilful technical assistance has also been provided by Mrs *Inger Ersman* Miss *Sonja Pettersson* and Miss *M L Sundin* This work has been supported by the *Swedish Board for Technical Development* (Grant No 68–335–f) and by *Emil och Wera Cornells stiftelse*

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Since the yields for most of the exoproteins were similar in continuous and batch culture a continuous process for large-scale production should be considered Small culture vessels with capacities of 1 10 l can be used The control of foaming aeration conditions and pH is easier in this scale and it is also to be perferred for the handling of pathogenic micro organisms Harvesting which is a serious problem with pathogens on a larger scale is much simpler in a continuous process

For the start of a continuous culture, batch growth was allowed to proceed to a bacterial density where the rate of enzyme formation was maximal When the continuous feed was started with a dilution rate of $0.5 h^{-1}$ the toxins and enzymes continued to increase for 2-4 h Then the activities were constant except for hyaluronate lyase and lipase The hyaluronate lyase activity decreased gradually during the whole 36 h run whereas lipase increased during the first 20 h and then remained constant (Fig 2) *Rogers* (12) observed an initial increase in the rate of hyaluronate lyase synthesis during the first hours of continuous growth of staphylococci This increase corresponds however to the initial increase in all the enzymatic activities obtained also in our experiments The slow decrease in the rate of hyaluronate lyase synthesis and the increase in the rate of lipase synthesis which followed must be ascribed to the specific environmental conditions in the continuous culture *Coulter* (6) observed that mutants with low α toxin producing

capacity replaced the original high producing strain in continuous cultures of strain Wood 46 Only 5 per cent of the original titre was obtained after a cultivation period of 2 weeks

A certain variation in the levels of α toxin, leucocidin, DNase phosphatase, and staphylokinase weren often experienced during a continuous run The bacterial density, on the other hand was generally constant Inactivation of the extracellular proteins in the foam was probably one the reasons for this variability, since the addition of the chemical foam breaker was performed intermittently

Another disturbance of the steady state also occurred if the foam control did not function properly Since an internal overflow was used, the culture volume decreased if a layer of foam of increasing depth was permitted When the foam was then broken by addition of the antifoam some time lapsed until the liquid level had risen sufficiently for the culture overflow to function once again Therefore automatic addition of the antifoam agent and an external levelling device should offer great advantages in this respect

Lysis of the bacteria was probably the main cause of the foaming The quantitative estimation of lysis in a growing culture in a complex medium is difficult The amount of intracellular proteins released into the culture liquid probably varied between cultures of different strains since the tendency to produce foam varied For this reason, the possibility must be considered that some of the enzymes or part of an enzymatic activity may not have been truly extracellular (11) Therefore washed bacteria were disintegrated by freezepressing (8) and the supernatants after disruption were tested for enzymatic activities The activities found were very low for all enzymes except lipase and staphylokinase The significance of the fact that part of these activities were cellbound under the conditions of cultivation used is presently under investigation

Our thanks are due to Miss *Berit Lindholm* for taking great interest in the development of the cultivation techniques Skilful technical assistance has also been provided by Mrs *Inger Ersman*, Miss *Sonja Pettersson* and Miss *M L Sundin* This work has been supported by the *Swedish Board for Technical Development* (Grant No 68–335 f) and by *Emil och Wera Cornells stiftelse*

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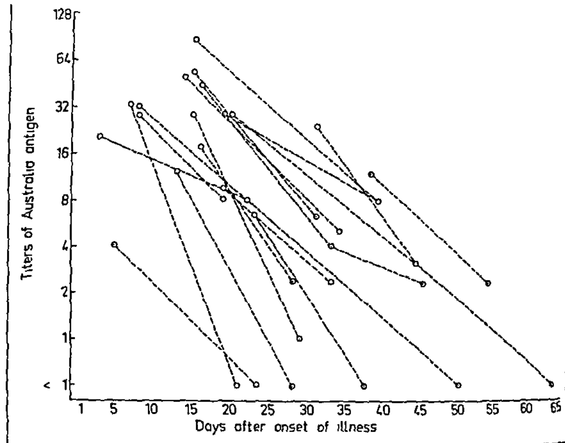


Fig 2 Australia antigen titres in paired serum specimens. *Titres are related to time after onset of clinical* symptoms

the disease The disappearance of Australia antigen within 54 days after onset of clinical symptoms is in accordance with findings in other studies (7 9), and differs markedly from results seen among patients subjected to haemodialysis (3 13 15) In these latter patients the frequency of carriers is high

In a previous study (19) it was pointed out that the frequency of secondary cases was higher in the outbreaks associated with haemodialysis than in the outbreaks among the track-finders Preliminary studies have suggested that the patients subjected to haemodialysis and subsequently developing an Australia antigen carrier state, which has persisted for several years have high Australia antigen titres in the range of 128 (3) *i e* significantly higher than the titres found in the track finders *i e* about 16 in the initial phase

The higher rate of secondary cases among haemodialysis patients may not only be due to the longer period of time this category of patients probably harbour an infectious agent, but may also be due to a higher concentration of an infectious agent in the blood of these patients (1)

The present data do not permit any conclusions concerning the frequency of a possible carrier state following hepatitis in track finders Epidemiological evidence *e g* the occurrence of a new outbreak when the preventive measures were abolished a few years after the epidemic (18) might suggest that carriers were present among the track finders at this time Evidently the present material is not large enough to permit the detection of a low rate of carriers In 47 out of the 69 patients studied only a single serum specimen has been tested and in the remain

ing 22 patients the second serum specimen was in several cases taken within a rather short time after onset of illness Only 14 serum specimens were drawn later than the 54th day after onset These were all negative

Attempts are presently made to collect sera from the majority of the track finders who had hepatitis in 1957–1966 in order to study whether these individuals become chronic carriers of Australia antigen at a frequency higher than that in the normal population without a history of hepatitis

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Acta path microbiol scand Section B 79 428–434, 1971

REPORT ON A PSEUDOLYSOGENIC MYCOBACTERIUM AND A REVIEW OF THE LITERATURE CONCERNING PSEUDOLYSOGENY

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A description is given of a rapidly growing mycobacterium (designated C U) which produces bacteriophages spontaneously The strain was isolated from an abscess that occurred 2 months *after a BCG vaccination The phage content was maintained after subculture* Since only some of the colonies produced phages and the content of phages could be eliminated after growth in antiserum against the phage (designated Bk_8) it is concluded that C U *is a pseudolysogenic mycobacterium No phage mediated changes in the charac*teristics of the bacterium were demonstrated The literature and various forms of pseudolysogeny are reported

According to the existing literature, pseudo lysogeny is not a single, well defined concept However, the condition can be distinguished both from lysogeny and from the lytic phage infection of bacteria In pseudolysogeny the DNA of the bacteriophage is not integrated with the DNA of the bacterium and not all bacteria contain the DNA It is possible to cure the bacterial strain of the phage by means of culture in antiserum against the bacteriophage or by isolation of non phage producing single colonies The bacteria can not be induced by the methods used in lysogeny

Pseudolysogeny was distinguished from lysogeny as early as 1925 (*McKinley* 1925), but interest in the phenomenon seems to have been relegated into the background by research into lysogeny Apart from one short paper (*Vinet et al* 1968) the recent literature mentions only pseudolysogeny *in experimentally* induced conditions

The present study deals with a naturally occurring pseudolysogenic mycobacterium Since the interest in this phage host relationship probably will increase among other reasons because of the demonstration of phage-mediated changes in the characteristics of bacteria of pseudolysogenic strains a survey has been made of the literature available on the subject

MATERIAL AND METHODS

Bacterial Strains

The rapidly growing *bacteriophage-producing* mycobacterium C U *was classified by Beck* in 1965 as *M fortuitum* (*Beck* 1965) It was isolated from

Received 11 xii 70

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an abscess near the site of a BCG vaccination performed 2 months earlier The strain was received by the writer from the Tuberculin Department, Statens Seruminstitut Copenhagen, where it had been maintained from 1963 to 1969 on subcultures on Lowenstein Jensen medium

The test strain C U $_4$ for the bacteriophage BK_5 (which was isolated from C U) was a subculture of a non phage producing single colony of C U It was not possible to demonstrate phages in repeated subcultures of C U $_4$

The mycobacterial strains used for determination of the host range of the bacteriophage were 9 strains of *M tuberculosis* isolated from routine material received at the Tuberculosis Department Statens Seruminstitut and 43 strains of rapidly growing mycobacteria the majority of which were received from the ATCC These consisted of 10 strains of *M smegmatis* 11 strains of *M fortuitum*, 12 strains of *M vaccae* and 10 strains of *M phlei*

Media

Lowenstein Jensen medium was used for maintenance of the bacterial strains and 7H10 agar for determination of the colony morphology of C U and C U $_4$ The bacteria were grown in Dubos fluid medium before inoculation of the petri dishes Bordet Gengou agar base with proteose peptone (*Froman et al* 1954) and a semi fluid medium with yeast extract and tryptone (*Froman & Scammon* 1964) were used in the experiments with the bacteriophage The phage was suspended in heart infusion broth to which was added 1 per cent tryptose 0 5 per cent NaCl and 0 001 M $CaCl_2$

Concentrations of bacteria and phages and inoculation of petri dishes

The agar layer method was used for demonstration of the bacteriophage and determination of its host range Inoculation of the plates was made with 0 3 ml of an exponentially growing culture of the rapidly growing mycobacteria in Dubos fluid medium the concentration of which was determined visually The inoculum of the *M tuberculosis* strains was 1 ml of a culture of the same concentration and growth phase For determination of the host range of the bacteriophage, drops of concentrations 10^8 pfu/ml and RTD were used for the rapidly growing mycobacteria and drops of ten fold serial dilutions from 10^9 to 10^7 pfu/ml for *M tuberculosis*

Demonstration of Bacteriophage Production in Single Colonies of Mycobacterium C U (Fig 1)

The bacteriophage isolated produced turbid lysis on the bacterium C U The bacteria from the growth in an area with confluent lysis on a Bordet Gengou agar plate were inoculated into Lowenstein Jensen medium in order to achieve growth of single colonies From ten of these colonies, ten tubes of Dubos fluid medium and ten Lowenstein Jensen plates were inoculated by a technique ensuring growth of single colonies The filtrates of the fully-grown Dubos cultures were tested for phages

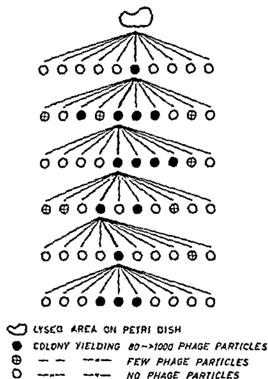


Fig 1 Content of phages in colonies of strain C U through five subcultures from one phage yielding colony

fluid medium and ten Löwenstein Jensen plates were inoculated from single colonies This process was repeated through five subcultures, each time from a Lowenstein Jensen plate corresponding to a phage producing colony

Production of Antiserum against the Phage

4 × 10^9 pfu were injected intravenously into a rabbit twice weekly for 6 weeks The serum was subjected to sterile filtration and stored in refrigerator at 4°C The K value was determined to be 4 4 min 1

Removal of the Bacteriophage from the Bacterial Strain by Means of Antiserum

C U was grown in a medium consisting of equal parts of heart infusion broth and phage antiserum. Three successive subcultures were made in the same medium. As control the bacteria were subcultured similarly in heart infusion broth without antiserum. The filtrates from the resulting eight cultures were tested on Bordet Gengou agar plates with C U_4 as test strain. From each of the four cultures grown in medium containing antiserum ten successive subcultures were made on Lowenstein Jensen medium. These 40 subcultures were inoculated into Dubos fluid medium and the filtrates of the fully grown cultures were also tested for phage content on the bacterium C U_4

Methods for Demonstration of Possible Differences between C U and C U_4

The methods mentioned under Results for examination of strains C U and C U_4 ie phage typing and acid production from carbohydrates were described by *Baess & Weis Bentzon* (1969). The iron uptake method was reported by *Szabo & Vandra* (1963) and the other biochemical tests and resistance determinations by *Engbæk et al* (1967)

RESULTS

Isolation of the Bacteriophage and Description of Plaques

A Bordet Gengou agar plate inoculated with 0 3 ml of a Dubos fluid culture of the rapidly growing mycobacterium C U was studded densely with turbid plaques from just visible to 1 5 mm in size. In order to ensure that this was not due to inadvertent laboratory infection, the experiment was repeated with bacteria from a new tube received from the Tuberculin Department. The result was the same. A bacteriophage designated BK_1 with C U_4 as host strain was isolated and concentrated from these plaques (Fig 2). The plaques on C U_4 had the same appearance as those that occurred spontaneously on C U. They were from just visible to 1.5 mm in size, bowl shaped and turbid though with slightly clearer central areas. The concentration of free plaque forming units in a 2 day-old Dubos culture of C U was 10 pfu/ml

Demonstration of Bacteriophage Production in Single Colonies of Mycobacterium C U

Examination of the bacteriophage production from single colonies by means of five subcultures from colonies containing the phage (Fig 1) revealed that there were 80 — >1000 plaques per plate in from 10 to 40 per cent of the colonies; there were no phages in 30 to 90 per cent; the remaining colonies (0 to 30 per cent) produced only few phages (1 to 13 plaques per plate). There were no plaques on the control plates without culture filtrate



Fig 2 Mycobacteriophage BK_1. Isolate with titre approximately $10^{?}$ pfu/ml concentrated ten times by centrifugation in Spinco ultracentrifuge at 40 000 r p m for one hour. Negative staining with 1 per cent ammonium molybdate. Magnification 90 000 ×

Removal of the Bacteriophage from the Bacterial Strain by Means of Antiserum

There were no phages in filtrates from four successive subcultures of C U in medium containing antiserum against the bacteriophage BK_5, while the petri dishes inoculated with bacteria from the control tubes were studded densely with plaques However, there were a few phages in the third subculture on Lowenstein Jensen medium without antiserum from the first subculture in antiserum The number of phages increased with each subculture until the plate from the filtrate of the sixth subculture was again studded densely with plaques No phages were found in ten subcultures from the second to the fourth subculture in phage antiserum Thus, the bacterium must be cultured twice in medium containing antiserum in order to be cured permanently of the phage

Attempts to Demonstrate Possible Differences between C U and C U₄

The phage-producing bacterium C U and its phage free variant C U.₄ were examined with a view to ascertaining possible differences Both bacteria consisted of acid and alcohol fast rods with the same appearance The colony morphology on 7H10 agar plates was the same viz dome shaped without flattened area at the beginning but later with a narrow, flat peripheral zone The colonies had smooth margins and smooth surfaces and were greyish white in colour One flat, rough colony of C U was phage susceptible and produced no phages This was interpreted as an incidental mutant lying outside the sphere of the present study

Both bacteria grew rapidly at 22° and 37° but not at 45° and 52°C They were lysed by BK_1 but not by phages D11, BK_5 D4 or D30 (*Baess* & *Weis Bentzon* 1969) Both produced acid from glucose, mannitol and inositol but not from rhamnose xylose, arabinose, galactose sorbitol dulcitol lactose maltose, and trehalose The bacteria split acetamide carbamide nicotinamide pyrazinamide, allantoin formamide and propionamide

The other biochemical tests also gave the same results with both bacteria, viz catalase test ++++, phenolphthalein sulphatase test +++ in 3 days, nitrate reduction +, niacin test —, Tween degradation —, iron uptake +

Resistance determinations were performed against streptomycin, para amino salicylic acid, isoniazid, thiosemicarbazone, ethionamide, pyrazinamide viomycin, cycloserine, acromycin, chloramphenicol, penicillin, sulphathiazole, sulphadiazin, kanamycin, rifampicin, ethambutol, capreomycin prothionamide, and disoxyl Both strains were resistant to all these drugs to the same degree

Examination of the Host Range of the Bacteriophage

Examination of the host range of the bacteriophage on 10 strains of *M smegmatis*, 11 strains of *M fortuitum*, 12 strains of *M vaccae*, 10 strains of *M phlei*, and 9 strains of *M tuberculosis* showed that all strains were resistant to BK_5

DISCUSSION

The phenomenon pseudolysogeny has long been known Descriptions given by *Twort* (1915) and *d Herelle* (1922) of the bacteriophages discovered by them indicate that their bacterial strains were pseudolysogenic *Gildemeister* (1917) demonstrated bacterial strains with circular defects in their colonies, and *Pfreimbter et al* (1922) found bacteriophages in the filtrates of those strains

As early as 1925 distinction was made between lysogeny and pseudolysogeny *McKinley* (1925) prepared antiserum against bacteriophages and was the first to use it to distinguish between what he called the spontaneous (now known as lysogeny) and the provoked (now known as pseudolysogeny) forms of lysogeny *Delbruck* (1946), in his review of bacteriophages, where he distinguished between true (lysogeny) and apparent (pseudolysogeny) lysogeny, gave as explanation for pseudolysogeny that the sensitive

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Acta path microbiol scand Section B 79, 435–436, 1971

BRIEF REPORT

NEISSERIA GONORRHOEAE COLONY VARIATION II

*A Reyn A E Jephcott** and *H Rain*

In a previous communication (1) two of us confirmed the existence of the four different characteristic types of gonococcal colony reported by *Kellogg et al* (2 3) In addition a fifth type was described It was also suggested that the various appearance of the colonies (when viewed with a stereomicroscope with oblique substage illumination) resulted from the ways in which each type reflected and refracted transmitted light

In the present investigation the shapes of these characteristic colony types were deduced from the profiles of vertical sections cut through the colonies in as near as possible to the vertical plane

Pure cultures of all available colony types from three strains of gonococci (F62 Kellogg 82409/SS 1969 43562/SS 1944) were grown on plates of Kellogg s Medium as described previously (1) After 16–18 hours incubation suitable examples of each colony type were exposed to vapour from a 3 per cent aqueous solution of glutaraldehyde for 1 hour and then covered with a layer of agar Carbon black was incorporated into this overlay agar to permit subsequent orientation of the colonies

Small agar cylinders each containing one colony were punched out of the plates and further treated in solutions of 3 per cent glutaraldehyde 1 per cent osmic acid and 2 per cent uranyl acetate This was followed by dehydration and embedding in Vestopal W (5) Thin sections were prepared on an LKB-ultrotome microtome with a view to electron microscopy (results in part reported**) and in addition 1 2 μ sections were cut at intervals throughout the colonies These sections were stained with toluidine blue and mounted on glass slides for examination with a conventional light microscope, they were only considered to be usable if there was a close correspondence between the heights and widths of the sections cut just each side of the maximum colony diameter

Type 1 colonies (Fig B) Sections revealed that these colonies were raised in the centre and that their edges merged gradually into the surface of the medium so that their upper and lower surfaces met at very sharp angles The domed centres could account for the considerable degree by which substage light was deviated by the colonies and the thin periphery merging gradually onto the medium could produce the soft appearance of the edge and the blurring of the highlights The sections had a slight granularity The lower limitation of the colonies tended to indent the medium

Type 2 colonies (Fig A) These colonies were quite different Each section had an asymmetrical oval outline The upper surface was domed and the lower was also convex in one strain though to a lesser degree producing a marked depression in the medium This indentation could also be observed macroscopically when colonies were scraped off the medium The angles where the upper and lower surfaces joined were smoothly rounded as they are in a drop of mercury This rounded drop like shape could well be responsible for the very sharply defined and refractile appearance of these colonies The granularity of the sections was more marked than those from type 1 colonies and the granules appeared to be arranged in layers

Types 3 and 4 colonies (Figs C and D) These were flatter than colonies of types 1 and 2 and the angles at their peripheries were very acute The relative flatness of these colonies could explain the much weaker effect that they had on transmitted light and consequently their less striking appearance Type 3 and type 4 colonies differ

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Studies carried out in Copenhagen during *A E Jephcott s* visit as a WHO Research Training Fellow

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** See brief report this issue

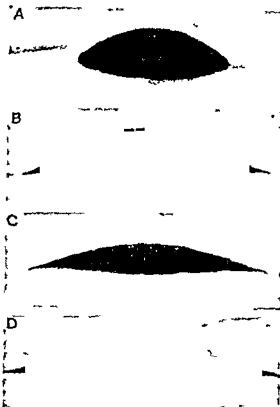


Fig A Midline vertical section of colony Strain F62 Type 2 Magnification × 185

Fig B Midline vertical section of colony Strain F62 Type 1 Magnification × 185

Fig C Midline vertical section of colony Strain F62 Type 3 Magnification × 185

Fig D Midline vertical section of colony Strain F62 Type 4 Magnification × 185

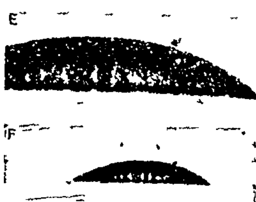


Fig E Midline vertical section of colony Strain 43562/SS 1944 Type 5 Magnification × 185

Fig F Midline vertical section of colony Strain 43562/SS 1944 Type 5 Magnification × 75

in that those of type 3 show internal granularity and are darker than type 4 colonies The granularity was very pronounced in the sections of type 3 colonies

Type 5 (Figs. E and F) Sections of these colonies showed that their outlines were a little more domed and more irregular than those of type 3 colonies but were otherwise similar The internal structure was coarsely granular and the cocci seemed less densely packed than in other types Vertical fissures were also seen these were not seen in other types and they may be responsible for the dark concentric rings sometimes visible in type 5 colonies

The profiles that we observed were a little different from those drawn by *Kellogg et al* (3) although these authors also considered that the edges of type 2 colonies were vertical and differed from those of type 1, and that types 3 and 4 were larger and flatter than types 1 and 2 The shapes

the different colonial appearances

Other phenomena, such as presence of capsules or slime and the packing of bacteria (4) can also affect colonial morphology No distinct capsules were detected by *Kellogg et al* (3) in any of the four characteristic colonial types and while by electron microscopy we have observed relatively small amounts of interbacterial substance predominantly in type 1 and 2 colonies (brief report this issue), we do not feel that this could *per se* account for the differences seen Similarly the regular layering of the cells at the type 2 colony might have some effect on transmitted light but again we feel that no such effect need be postulated to explain the colony's appearance

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Acta path microbiol scand Section B 79, 437–439, 1971

BRIEF REPORT

NEISSERIA GONORRHOEAE III
DEMONSTRATION OF PRESUMED APPENDAGES TO CELLS FROM DIFFERENT COLONY TYPES

A E Jephcott, A Reyn* and *A Birch Andersen*

It has become apparent that gonococci of the same strain can give rise to several colonial forms (5, 6, 7) Further there appears to be a correlation between colony type and virulence (6, 7) Two types of colony (types 1 and 2) are to be found in varying proportions in primary cultures of acute gonorrhoea, whereas three other types arise on subculture (types 3, 4, 5)

In the present study the fine structure of members of each colony type was examined in the electron microscope to see if any differences could be detected It was thought possible that any differences observed might be relevant to the virulence of gonococci

In order to preserve the gonococci in as near as [illegible] colony whole colonies

[illegible]

magnesium uranyl acetate and lead citrate, and examined

The study of intracolonial organisms revealed cell envelopes of the appearance now associated with gram negative organisms, viz an inner cytoplasmic membrane of the unit membrane type, an intermediate rather dense layer and an outer, crenated, three layered membrane The internal structure of the cells was also unremarkable

No significant differences could be detected either between the cells of different colony type or between cells of different parts of the same colony However, electron dense material was seen lying in patches between the cells of colony types 1 and 2, but no such material was visible in types 3, 4 or 5 The patches were up to several microns in length and could be seen at relatively low magnification (Fig 1) At higher magnification (Fig 2) they were seen to consist of bundles of fibrils lying side by side with centre to centre distances of 5 5 nm Cross sections of the bundles often revealed that the fibrils were regularly aranged in groups of five (Fig 3 arrows)

Further, fibrils could be visualized by negative staining of suspensions of live gonococci with 1 per cent ammonium molybdate Even in such specimens they were seen frequently in large bundles (Fig 4) which suggested that bundle formation was not an artefact produced during the fixation necessary for sectioning With the negative staining technique the fibrils could be examined with greater ease, and a survey of many specimens with a crude quantitation was carried out

Fibrils were found to be most abundant with cells from type 2 colonies and were detected in all specimens examined of these and of type 1 colonies They could be found only in small numbers or were entirely absent from almost all colonies of types 3, 4 or 5 They tended to be straight or gently curved Sharp kinks were rare No hollow [illegible]

[illegible]

woven nets but commonly rather tightly packed in bundles In some parts of a field of view two or more bundles would cross each other In such places the fibrils appeared interwoven (Fig 5 arrow) In yet other parts twisting of whole bundles was evident.

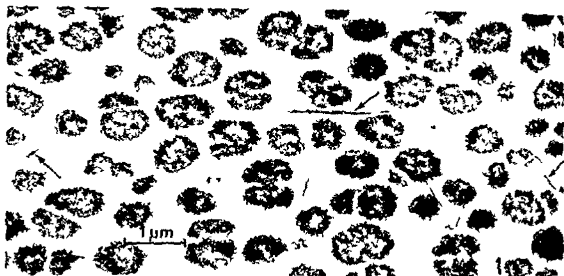
Occasionally a bacterial cell was found from which fibrils appeared to be protruding but the drying of the specimens after negative staining may

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Received 30 iii 71 *from The Neisseria Department (WHO International Reference Centre for Gonococci) and the Department of Biophysics, Statens Seruminstitut, Copenhagen, Denmark*

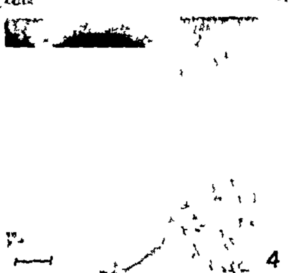
Requests for reprints should be addressed to Dr *Alice Reyn*, Neisseria Department Statens Seruminstitut, Amager Boulevard 80, 2300 Copenhagen S Denmark

*Present address Public Health Laboratory, Sheffield England Studies carried out in Copenhagen during A E Jephcott s visit as a WHO Research Training Fellow


1 μm
1


2


3


4


5

All material from type 2 colonies of strain SS 82409/1969 Unless otherwise stated the bar on each micrograph represents 0 1 µm

Fig 1 Section through colony Note electron dense patches between *cells* (arrows) *Magnification* 15 000×

Fig 2 Detail from section through colony, showing bundle of longitudinally cut fibrils Magnification 170,000×

Fig 3 Detail from section through colony Note honeycomb pattern of cross sectionned bundle of fibrils (arrow) Inset shows single group of five fibrils (arrow) Magnification 170,000×

Fig 4 Dividing gonococcus with large bundle of fibrils apposed to cell Negative stain, 1 per cent ammonium molybdate Magnification 90,000×

Fig 5 Detail from twisting bundles of fibrils Note crossing bundles (arrow) Negative stain, 1 per cent ammonium molybdate Magnification 160,000 ×

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have introduced artefacts, and until now no unequivocal evidence for a direct connection between cell wall and fibril has been found Despite this we still feel that the fibrils most closely resemble the fimbriae (or pili) of gram negative rods (1 2 4)

It is possible that the fibrils may play some part in the virulence of the gonococcus, but we have seen similar fibrils in preparations of other gram negative bacteria, including meningococci, both from primary cultures and from old laboratory *strains* Somewhat similar fibrils were also described by *Bøvre et al* in certain colony types of *Moraxella* (3)

At neutral pH fibrils will remain dispersed in water but at pH 3 to 4 iso-electric aggregation occurs This has allowed some degree of purification Analysis of these impure suspensions suggests that the fibrils contain at least some carbohydrate, but that protein and lipid are not major components

References 1 *Brinton, C C Jr* Trans N Y Acad Sci *27 1003-1034, 1965*—2 *Brinton, C C Jr* In The specificity of cell surfaces (Davis B D & Warren, L, Editors) Prentice Hall Inc Englewood Cliffs, New Jersey, 1967, p 33-70—3 *Bøvre, K, Bergan, T & Frøholm, L O* Acta path microbiol scand Section B, *78* 765-779, 1970—4 *Duguid, I P & Campbell, I* J med Microbiol *2* 535-553, 1969—5 *Jephcott, A E & Reyn, A* Acta path microbiol scand Section B, in press—6 *Kellogg D S Jr, Peacock, W L Jr, Deacon, W E, Brown, L & Pirkle, C I* J Bact *85* 1274-1279, 1963—7 *Kellogg, D S Jr, Cohen, I R, Norins, L C, Schroeter, A L & Reising, G* J Bact *96* 596-605, 1968—8 *Reyn, A, Jephcott, A E & Ravn, H* Acta path microbiol scand Section B, 79 435-436 1971

Acta path microb ol scand Sect on B 79 440–448 1971

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting 26 28th November 1970

Margareta Lagercrant Department of Virology Statens Bakteriolog ska Laboratorium Stock holm EVALUATION OF CROSS REACTIONS WITHIN THE ENTERO VIRUS GROUP USING NEUTRALIZA TION TESTS

Neutral zing ant bod es aga nst Coxsack ev rus types B 1-6 A7 A9 ECHO v rus types 1 3 4 6 7 9 11 14 16 17 25 and Pol o irus types 1 3 were determ ned in pa red sera from pat ents from I om the respective types of v rus had been isolated Serum d lut ons n 4 fold steps ere incorporated n agarose med m into series of petr dishes w th un nfected cultures of GMK cells The 22 v rus types were appl cated in f lterpaper d scs on the agar layer After incubat on and sta n ng plates were read for cytopath c degenerat on zones Absence of a zone was taken as neutral zat on of the respecti e v rustype

Of 42 paired sera 36 showed a homologous t tre rise 4 fold or h gher E gh teen pa rs showed no heterologous rise of t tre 12 sho ed one 7 pa rs showed ? and 5 had 3 For 3 serum pa rs (anti B1 B3 B5) the heterologous rise of t tre was h gher than the homologous For further 3 pa rs (anti B4 E4 E17) the heterologous t tre rise was as h gh as the homologous Among the 715 poss ble heterologous comb nat ons 41 heterologous rises of ... were lower ... the homologous Cross d cro s ngs seemed to be randomly d stributed among the var ous v rus types

Mon ca Grand en B th Ch istensson & I Esp mark Department of V rology Statens Bakteriologiska Laborator um S ockholm IMMUNO DIFFUSION TESTS FOR ANTIBODIES TO HERPES SIMPLEX (H S) VARICELLA ZOSTER (V Z) AND CYTOMEGALO VIRUSES (CMV)

The a m of the st dy was to eval ate the poss ble usefulness of the immunod ffu on test (ID fo the diagnos s of nfect ons ca sed by the various members of the herpesv rus group

Pa red sera from 69 pat ents I s gn ficant rises in CF t tres aga nst e ther H S V Z or CMV vere tested by ID against the homologous ant gen A d stinct ncrease n the ID rea ion occurred n 55 pat ents equal prec p a on of he two sera was found n 13 a d only n one pa ient ere both sera negat e — Among 50 normal sera t sted n the same way about 40 per cent. g e eak react ons v h each of the an gens CF ant bod es mostly i i low ti res were demons rab e n 46 per cent The CF and ID test ere concordan in 68 per cent of the cases

Out of 68 sera tested quant a ely for an *bod es to CMV by a rad al immunod ffusion* (RID) tech nique all 53 w th CF t res exceeding 10 were pos t e hereas 5 sera h CF t es less than 5 were negat ve Under the cond on used a t o-fold change n CF t re corresponded to 1 mm change of the d ame er of the prec p a on zone

S Holont s & B Bjorvatn Dep of V rology S ukvårdsstyrelsens Bakt Centrallab S ockholm I EXPERIENCES OF ISOLATION FROM CEREBROSPINAL FLUID AND URINE II ATTEMPTS AT EARLY ROUTINE ... OF MUMPS ... TION

To be publ sh ed n Scand J Inf Dis

Bengt Go an Hans on To n John on and C O K ndma k Dept of Cl V rol and Dep of Inf D seases Un ers v I nd Malmo G... BL... AND ... ANTIGEN

The sens t ty of the immunoel c roos ... (IFOP) method performed n agar-agarose and pure agarose gel was compa e l w h the Ou

lony gel-diffusion method in detecting Australia (Au) antigen The IEOP method with agar-agarose gel gave after staining the plates about 6 times higher antigen titres than the Ouchterlony method Performed with pure agarose gel the antigen titres with the IEOP method were about 10 times higher before staining and 21 times higher after staining the plates, compared to the Ouchterlony method In a study of 20 Au positive cases with consecutively drawn blood samples 50 per cent of the patients were still positive 35 days after onset of icterus, when using IEOP with pure agarose and staining the plates The corresponding figure was only 23 days, when using the Ouchterlony method In the total material of suspected cases of hepatitis there where 10 per cent more positive patients with the IEOP method using agarose and staining the plates than with Ouchterlony method When more than one serum from the Au positive patients were included in the material, 27 per cent more positive sera were found

Margareta Böttiger, B Zetterberg & C R Salenstedt, Dept of Virology, Karolinska Institute and National Bacteriological Laboratory, Stockholm SERA IMMUNITY TO POLIO MYELITIS IN SWEDEN 10 YEARS AFTER INTRODUCTION OF GENERAL VACCINATIONS WITH INACTIVATED POLIO VIRUS VACCINE

To be published in Acta Path Microbiol Scand Section B

Gunnel Huldt & S Gard, National Bact Lab and Dept Virol Karolinska Inst, Stockholm, EXPERIMENTAL INFECTION OF NEWBORN MICE WITH TOXOPLASMA AND MOLONEY VIRUS

Newborn mice C57Bl or CBA were inoculated with either Tx, MLV, or both agents

Runting occurred in 30 per cent of mice receiving only Tx and was accompanied by cortical atrophy of thymus and decrease in lymphocytes in germinal centres of lymphatic organs Nephritis and immune complexes containing Toxoplasma antigen were demonstrable histopathologically Sixty nine per cent of the mice died before the age of 8 weeks as compared to 9 per cent among untreated controls

With MLV alone moderate runting was seen in 5 per cent Changes in the lymphoid cell series within the first 8 weeks were restricted to slightly increased nuclear staining of reticuloendothelial spleen cells Twenty three per cent of the animals died before the age of 8 weeks.

When both agents were given together almost all animals runted Ninety five per cent died within 8 weeks Small lymphocytes had virtually disappeared, only small scattered groups of blast cells were seen leaving the stromal network of spleen and lymph nodes exposed Nephritis with immune complexes was demonstrable

The results suggest a synergistic effect of the two agents

E Norrby, Dept of Virology, Karolinska Institute, School of Medicine, Stockholm THE EFFECT OF A CARBOBENZOXY TRIPEPTIDE ON THE BIOLOGICAL ACTIVITIES OF MEASLES VIRUS

To be published in Virology

Göran Wadell, Department of Virology, *Karolinska Institutet, School of Medicine, Stockholm* IN VITRO NEUTRALIZATION OF INFECTIVITY OF ADENOVIRUSES BY SPECIFIC SERA AGAINST CAPSID COMPONENTS

Antisera against hexons of members of subgroups I and II and serotype 4 consistently displayed high —and sera against fibres or pentons of all tested serotypes low—ratios of neutralizing capacity over typespecific CF activity Two different populations of hexons of serotype 2 (subgroup III) could be separated by anion exchange chromatography and isoelectric focusing Sera against certain preparations of hexons obtained by these techniques displayed very low neutralizing activity in spite of the presence of high amounts of type specific CF antibodies

Using heterologous anti antibodies the neutralization by sera against hexons of members of subgroup III (except type 4) was enhanced Furthermore a *dramatic enhancement* of the neutralizing capacity of sera against fibres or pentons of all subgroups was noted This neutralization was not due to aggregation of virions, since it appeared after adsorption of virions to the cell surface Fab and $F(ab)_2$ fragments of virusspecific antibodies, which were devoid of neutralizing activity, retained capacity to interact with antigenic determinants on fibres and hexons as demonstrated by an antibody blocking test based on CF Combined with anti antibodies these virus specific antibody *fragments* were capable of neutralizing the adenovirus infectivity indicating that Fc fragments of virus specific antibodies were not required for this kind of neutralization

J Ankerst, Dept Med Microbiol, *University of Lund* DEMONSTRATION AND IDENTIFICATION OF CYTOTOXIC ANTIBODIES AND OF ANTIBODIES BLOCKING THE CELL-MEDIATED ANTI TUMOUR IMMUNITY AGAINST ADENOVIRUS TYPE 12 INDUCED TUMOURS

To be published in Cancer Research

Acta path microbiol scand Section B 79, 440–448, 1971

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting 26–28th November 1970

Margareta Lagercrantz, Department of Virology, Statens Bakteriologiska Laboratorium, Stockholm EVALUATION OF CROSS REACTIONS WITHIN THE ENTERO VIRUS GROUP USING NEUTRALIZATION TESTS

Neutralizing antibodies against Coxsackievirus types B 1 6, A7, A9, ECHO virus types 1, 3, 4, 6, 7, 9, 11, 14, 16, 17, 25 and Poliovirus types 1 3 were determined in paired sera from patients from whom the respective types of virus had been isolated Serum dilutions in 4 fold steps were incorporated in agarose medium into series of petridishes with uninfected cultures of GMK cells The 22 virus types were applicated in filterpaper discs on the agar layer After incubation and staining plates were read for cytopathic degeneration zones Absence of a zone was taken as neutralization of the respective virustype

Of 42 paired sera 36 showed a homologous titre rise, 4 fold or higher Eighteen pairs showed no heterologous rise of titre 12 showed one, 7 pairs showed 2 and 5 had 3 For 3 serum pairs (anti B1, B3, B5) the heterologous rise of titre was higher than the homologous For further 3 pairs (anti B4 E4 E17) the heterologous titre rise was as high as the homologous Among the 715 possible heterologous combinations 41 heterologous rises of titres were lower than the homologous Observed crossings seemed to be randomly distributed among the various virus types

Monica Grandien Brith Christensson & A Espmark, Department of Virology Statens Bakteriologiska Laboratorium, Stockholm IMMUNO DIFFUSION TESTS FOR ANTIBODIES TO HERPES SIMPLEX (H S), VARICELLA ZOSTER (V Z) AND CYTOMEGALO VIRUSES (CMV)

The aim of the study was to evaluate the possible usefulness of the immunodiffusion test (ID) for the diagnosis of infections caused by the various members of the herpesvirus group

Paired sera from 69 patients with significant rises in CF titres against either H S, V Z or CMV, were tested by ID against the homologous antigen A distinct increase in the ID reaction occurred in 55 patients, equal precipitation of the two sera was found in 13, and only in one patient were both sera negative —Among 50 normal sera tested in the same way about 40 per cent gave weak reactions with each of the antigens CF antibodies mostly in low titres, were demonstrable in 46 per cent The CF and ID test were concordant in 68 per cent of the cases

Out of 68 sera tested quantitatively for antibodies to CMV by a radial immunodiffusion (RID) technique, all 53 with CF titres exceeding 10 were positive, whereas 5 sera with CF titres less than 5 were negative Under the conditions used a two fold change in CF titre corresponded to 1 mm change of the diameter of the precipitation zone

S Wolontis & B Bjorvatn Dept of Virology, Sjukvårdsstyrelsens Bakt Centrallab, Stockholm I EXPERIENCES OF ISOLATION FROM CEREBROSPINAL FLUID AND URINE II ATTEMPTS AT EARLY ROUTINE LABORATORY DIAGNOSIS OF MUMPS SOME EXPERIMENTS WITH IMMUNO-FLUORESCENCE AND HAEMADSORPTION

To be published in Scand J Inf Dis

Bengt Goran Hansson Torsten Johnsson and C O Kindmark Dept of Clin Virol and Dept of Inf Diseases University of Lund Malmo Ge ... BE

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ANTIGEN

The sensitivity of the immunoelectroosmophoresis (IFOP) method performed in agar-agarose and pure agarose gels was compared with the Ouchter

L Lindholm & L Rydberg Institute of Medical Microbiology Dept of Bacteriology University of Gothenburg Gothenburg Sweden DEVELOPMENT OF PLASMA CELLS DURING ACUTE GRAFT VERSUS HOST REACTIONS

Graft versus host reactions were induced by the injection of adult parental strain thymus— or spleen cells into two days old F1 hybride mice Light—and electron microscopy revealed the presence of rather large numbers of plasma cells in the lymphnodes of the injected animals whereas the lymphnodes of uninjected litter mates contained no such cells The plasma cells were demonstrated from the sixth day after injection of either thymus or spleen cells and were localized to the medullae and to a lesser degree to the paracortical areas of the nodes By means of an anti immunoglobulin allotype serum specific for host immunoglobulins it was possible to demonstrate that recipients of thymus cells but not recipients of spleen cells contained elevated levels of immunoglobulin of host origin Furthermore a certain proportion of the injected animals showed a positive direct Coombs reaction when tested with the same anti immunoglobulin serum These results suggest that graft versus host reactions induced by thymus cells leads to the development of plasma cells of host origin

A Tarnvik Dept of Clin Bact University of Umeå Umea INFLUENCE OF AUTOLOGOUS BLOOD CELLS ON THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ

Human peripheral lymphocytes were purified from other leucocytes and from red cells The purified lymphocytes responded poorly to phytohaemagglutinin (PHA) as measured by incorporation of ^3H thymidine into DNA The response was increased by addition of red cells or red cell membranes but not by soluble products from red cells A preparation containing mononuclear leucocytes and platelets (leucocytes platelets) increased the PHA response

PHA-coated red cell membranes stimulated purified lymphocytes The response was higher than that caused by an optimal concentration of PHA in soluble form

PHA was subjected to ion exchange chromatography One of the fractions obtained agglutinated red cells and leucocytes platelets the lymphocyte response to this fraction was increased by red cells as well as by leucocytes platelets Another PHA fraction agglutinated leucocytes platelets but not red cells the response to this fraction was increased by leucocytes platelets but not by red cells

The results indicate that a complex of PHA and red cell membrane has higher lymphocyte stimulating activity than has free PHA

Hans Ericsson & J C Sherris Dept of Clin Bact, Karolinska Hospital Stockholm Sweden and Dept of Microbiology School of Medicine, University of Washington Seattle Washington USA PROPOSALS FOR WHO STANDARDS FOR DETERMINATION OF BACTERIAL SENSITIVITY TO ANTIBIOTICS

To be published in Acta Path et Microbiol Scand

B Nystrom Central Laboratory for Clinical Bacteriology Karolinska Hospital Stockholm SENSITIVITY PATTERN IN BACTERIA ISOLATED FROM PATIENTS IN THE KAROLINSKA HOSPITAL IN 1969

To be published in Acta Path et Microbiol Scand

I Juhlin & Hoger Hansson Institute of Clinical Bacteriology and Department of Dermatology University of Lund 214 01 Malmo SENSITIVITY AND APPEARANCE OF RESISTANCE TO RIFAMPICIN IN N GONORRHOEAE

The IC_{50} values for rifampicin were lower than 1 0 mcg/ml in 600 strains of primarily isolated gonococci and 95 per cent were sensitiv to 0 35 mcg/ml Among 1300 primarily isolated cultures not one was found to be resistant to rifampicin

The concentration of rifampicin in blood after one single dose of 0 9 g was followed in 19 patients All showed concentrations above 4 0 mcg/ml blood and most of the patients showed values around or above 10 0 mcg rifampicin/ml 454 patients have been treated with 0 9 rifampicin orally in one single dose 39 with a positive control culture denied reinfection In 27 cases (6 per cent) the therapeutic failure could be explained by the appearance of gonococci resistant to rifampicin

The frequency of rifampicin resistant variants was studied in 25 primarily isolated cultures In 7 of these with a mean value of $1\,9\times10^9$ colonies 1 to 5 colonies resistant to rifampicin were found

In another study sub-cultures were made from one single colony In 3 out of 5 cultures with $3\,8\times10^9$ colonies between 1 and 5 colonies were found to be resistant to rifampicin

In 51 out of 62 cultures primarily sensitive to less than 1 0 mcg/ml the resistant variants showed IC_{50} values above 1000 0 mcg/ml

No cross resistance between rifampicin and benzylpenicillin or ampicillin was found

K Lincoln Inst of Med Microbiol Dept of Clin Bact University of Goteborg Goteborg TRANSFERABLE DRUG RESISTANCE OF E COLI ISOLATED FROM URINE AND FAECES OF CHILDREN

Ability to transfer resistance to sulphonamide (Su) tetracycline (T) chloramphenicol (C) strepto

mycin (S), or ampicillin (A) was shown in 295 (64 per cent) of 461 isolates of *E coli* cultured from urine and periurethral and rectal swabs from children with upper respiratory tract infection or urinary tract infection and from infants with myelomeningocele Most of the patients were or had been treated with sulphonamide, and were *or had been hospitalized*

The method of transfer was similar to that of Datta (Brit med J, 1969, 2, 407), but with two recipient strains simultaneously one lactose positive haemolytic *E coli* 06 and one lactose negative, non haemolytic *E coli* 04 The resulting resistant recipients were identified by O grouping

The most common of the 16 different resistance patterns encountered were Su (18), T (17), SuT (17), SuA (12), SuTCA (12), SuCA (6), A (4), and SuTCSA (4) The figures denote per cent of the 461 isolates

Fourteen different patterns could be transferred either as a whole or in part, *e g* SuTCA (82), SuCA (58), SuT (58), Su (55), SuS (33), T (33), SuA (16) SuTCSA (12), and A (12) The figures indicate per cent of isolates with this pattern which transferred the whole pattern

A female myelomeningocele infant died at the age of two weeks of a meningitis caused by *E coli* 04 SuCA The whole resistance pattern was transferable

Anna Hambraeus, Institute of Medical Microbiology Department of Clinical Bacteriology University of Uppsala Uppsala STAPHYLOCOCCAL INVASION INTO A NEW BURNS UNIT

The invasion of S aureus into a new burns ward at the University Hospital of Uppsala has been st idied since its opening Specimens obtained from the patients and the staff have been cultured once a week and from the patients on the day of arrival in addition Air contamination in the corridor and wardrooms has been studied using open plates The S aureus strains isolated have been identified by phage typing The spread of one strain type 84 which was methicillin resistent was chosen to illustrate the epidemiology in the ward This strain caused infection in 24 patients and was isolated from 27 members of the staff The infected patients were fairly evenly scattered over a 40 weeks period except for a two weeks period when 6 patients were infected This outbreak was preceded by a high carrier rate among the staff and a heavy air contamination in the wardrooms The infection rate among the patients decreased when the air contamination decreased Despite a high carrier rate among *the staff there was no new* outbreak among the patients Airborne transmission may not have been of any significance since there was only a slight increase in airborne bacteria in the corridor during the outbreak The findings might suggest that passive transfer of bacteria by the staff was a more important mode of transmission

C Henning, H Bucht L O Kallings Dept of Bacteriology, National Bacteriological Laboratory Stockholm and Sahlgrenska Hospital Gothenburg RESULTS FROM THE ROUTINE DETECTION OF BACTERIURIA IN ANTENATAL CARE IN STOCKHOLM COUNTY

Before introducing bacteriural screening as a general routine health test in public antenatal care a pilot study was performed in Stockholm County during 1969 on the practical possibilities of performing bacteriological screening and of recording and treating cases with significant bacteriuria

Each woman attending the antenatal care was *followed with quantitative bacteriological cultures* twice during pregnancy and once immediately after pregnancy

Every culture showing at least 10 000 bacteria per ml was repeated When two or more cultures were showing 100 000 bacteria per ml or more significant bacteriuria was supposed to be proven

All cases with bacteriuria were treated throughout pregnancy were controlled with urine cultures one to two weeks after started therapy and were also followed after delivery

The study comprised 6729 patients Bacteriuria was demonstrated in 3 7 per cent of the patients, E coli was the cause of bacteriuria in 77 per cent *and Staphylococcus albus in* 9 per cent 81 per cent of the isolated bacteria were sensitive to sulphonamide *99* per cent to *nitrofurantoin* and 98 per cent to ampicillin

When repeated a single isolation of 100 000 E coli per ml was shown to be due to significant bacteriuria in 67 per cent and a single isolation of 10 000 E coli per ml in 18 7 per cent. This showing the need of repeating urine culturing at least twice before giving the diagnosis bacteriuria and instituting treatment

Birgitta Nilehn Dept of Med Microbiol The Medical School Linköping SOME ASPECTS ON *PHAGE TYPING OF YERSINIA ENTEROCOLITICA*

Y entercolitica phages were first described by Mollaret & Nicolle (1965) Nicolle et al (1967) Nilehn & Ericson (1969) and Nilehn (1969) further evaluated phage typing of Y enterocolitica Nicolle et al (1967) described 9 typing patterns especially useful for separating strains of animal origin

In the present study 332 strains of human

origin belonging to the most common serotypes 3 or 9, were investigated concerning their sensitivity to 7 Y enterocolitica phages and one Y pseudo tuberculosis phage (Girard, ps 97/II)

Forty strains of serotype 9 were all non typable, while 292 strains of serotype 3 could be divided into 6 different types The majority of strains isolated in Scandinavian countries differed in their phage patterns from strains from other countries Two small groups of strains from Canada and South Africa, respectively each belonged to one distinct phage type

The host range of phage ps 97/II was influenced by propagation strain and propagation temperature After propagation at 25°C on strain ps 97/II, high titre phage showed lytic activity on many Y enterocolitica strains Propagation at 37°C on one Y enterocolitica strain of serotype 8, gave a phage active against some previously non typable Y enterocolitica strains of human or animal origins

Y enterocolitica phages otherwise used, preferably operated at 25°C, single systems studied, showed a gradually decreasing e o p and plaque size with temperatures increasing up to 30°C Inactivation of phage was not significantly higher at 37°C than at 25°C, nor seemed adsorption to be less at the higher temperature

T Wadstrom & *B Wretlind*, Dept Bact Karolinska Institute, S 104 01 Stockholm 60 and Dept Clin Bact, Karolinska Sjukhuset S 104 01 Stockholm 60 STAPHYLOCOCCLYTIC ENZYMES

The cell wall of *Staphylococcus aureus* is more difficult to break by mechanical methods and most bacteriolytic enzymes than walls from most other species However, during the last five years a few enzymes specifically attacking the staphylococcal cell wall have been isolated from *S epidermidis* strain K6 W1 (Lysostaphin®) and the genera *Flavobacterium*, *Aeromonas*, and *Pseudomonas* Analysis of strains of these species on agar plates containing whole cells of heat killed *S aureus* showed that 37 out of 58 (64 per cent) strains of *Pseudomonas aeruginosa* were producers, while 58 strains of *Pseudomonas fluorescens* and other *Pseudomonas* species were non producers 18 out of 28 strains of *Aeromonas hydrofila* all six fish pathogenic *Aeromonas* and five of nine strains of *Flavobacterium* species were producers of staphylolytic enzymes but none of the strains of *Aeromonas shigelloides* (14 strains) *Escherichia coli* (35 strains) *Klebsiella Enterobacter* (20 strains) Neither were twelve non fermentative Gram negative rods producers Lysostaphin and the staphylolytic enzyme from one strain of *Pseudomonas aeruginosa* have been separated by isoelectric focusing Both are basic proteins with isoelectric points of 10 5 and about 8 degrading the pentaglycin cross bridge of the staphylococcal peptidoglycan Further studies of *these specific staphylolytic enzymes are* in progress which all seem unable to degrade any other bacterial cell walls, except those from strains of *S aureus*

R Mollby & *T Wadstrom* Dept of Bacteriology, Karolinska Institute, Stockholm PURIFICATION AND BIOLOGICAL STUDIES OF STAPHYLOCOCCAL HAEMOLYSINS

To be published in Biochim Biophys Acta and Infection and Immunity

Paula Branefors Helander, Inst Med Microbiol, Dept of Bact, Goteborg CAPSULAR B AND SOMATIC ANTIBODIES AGAINST HAEMOPHILUS INFLUENZAE

Antibodies against Haemophilus influenzae substance b (anti Mb) were measured by the passive haemagglutination At the onset of acute epiglottis and meningitis due to H influenzae type b, anti Mb could not be demonstrated in 19 patients, whereas 3 14 days later anti Mb was detected in all patients The titres were low, 1 8 to 1 256, compared to the titres found in hyperimmunized rabbits but anti Mb could still be demonstrated in the patients one year later In about 8 per cent of 180 patients with acute otitis or sinusitis due to organisms other than H influenzae type b persistently low titres of anti Mb were noted

H influenzae O antibodies (anti O) were studied by the complement fixation test using a bacterial pool from 20 unencapsulated H *influenzae* strains as the antigen Two hundred control children, 0 16 years old were studied Anti O titres up to 1 60 were found in newborns, but not titres were demonstrated during the rest of the first year In older children, titres up to 1 60 were *found in* $^2/_3$ *to* $^3/_4$ *of the* children just as in patients with acute otitis or sinusitis from which H influenzae was not isolated Increasing anti O titres were seen in all patients with H influenzae type b infections (maximum 1 3840) and in most patients with acute otitis media where H influenzae was isolated from the nasopharynx Patients with acute sinusitis often had elevated titres already at the onset

K A Broholm & *J Holmgren*, Inst Med Microbiology University of Goteborg IMMUNO DIFFUSION STUDIES ON PNEUMOCOCCAL ANTIGENS WITH SPECIAL REFERENCE TO SEROTYPE 27

The antigenic patterns of 13 pneumococcal strains belonging to 6 *different serotypes all* capsulated except one were studied with immunoelectropho

resis and double diffusion methods A strain of serotype 27 was used as model strain and contained at least 21 antigenic factors of which 16 were demonstrated in the nucleoprotein fraction and 4 in the M protein preparation. The serotypes 2R 11S 26S and 29S lacked between 5 7 of the antigenic factors of the model strain A strain of serotype 3S lacked 12 of the factors In immunoelectrophoresis at pH 8 2 most of the factors of the model strain migrated towards the anodal side Double diffusion studies of capsular polysaccharides (SSS) prepared by fractionated alcohol precipitation and phenol extraction revealed the occurrence of contaminating C polysaccharide Within the pH range 4 9 the capsular polysaccharides showed an anodal migration negligibly increasing in rate with rising pH SSS 3 showed the fastest migration rate which increased significantly with higher pH The electrophoretic migration of the C polysaccharide varied with pH At pH higher than 7 the C polysaccharide showed no electrophoretic mobility At pH 4 5 it moved slowly towards the cathode This circumstance made it possible to obtain by preparative horizontal block electrophoresis capsular polysaccharide freed from C polysaccharide for use in e g complement binding and passive haemagglutination experiments

J Holmgren, I Lonnroth & O Ouchterlony, Inst of Med Microbiology University of Goteborg
IDENTIFICATION AND CHARACTERIZATION OF CHOLERA EXOTOXIN IN CULTURE FILTRATES OF *V CHOLERAE*

Two culture filtrates of *V cholerae* labelled 4493 G (Inaba) and 001 (Ogawa) were studied concerning toxicity (Craig method), precipitinogenicity and chemical composition The two lots although very different in chemical composition, contained three identical precipitinogens one of which was identified as type 2 cholera exotoxin Also the toxicity of the two preparations was very similar 4493 G and 001 both contained a fourth cross reacting precipitinogen related to lipopolysaccharide

The type 2 toxin active in the intradermal test identified completely in comparative double diffusion analyses with toxin active in the infant rabbit and the ileal loop models The isoelectric point of the toxin was found to be within pH 6-7 The toxicity was completely retained by an UM 10 membrane (cut at m w 10 000) practically completely retained by a PSFD membrane (cut at m w 25,000) but not retained by an XM 50 membrane (cut at m w 50 000) By gel filtration through agarose the type 2 toxin could be separated from the other more rapidly filtering three precipitinogens and was eluted in a volume corresponding to an m w of 25 000 38 000 while gel filtration through Sephadex G 75 indicated the size of the type 2 toxin to correspond to an m w of 55 000-60 000

Ann Mari Svennerholm J Holmgren & O Ouchterlony Inst Med Microbiology University of Goteborg
AN AGAR PLAQUE METHOD FOR QUANTITATION OF VIBRIOCIDAL ANTIBODIES

In epidemiological studies it has been shown that protection against cholera is directly correlated to the vibriocidal antibody titre in serum We have therefore developed a method for quantitation of such antibodies Droplets of a mixture of complement and serial dilutions of the serum to be tested are applied on the surface of a nutrition agar plate with incorporated live vibrios After incubation of the plate at 37°C for four hours the complement dependant vibriocidal effect of the antibodies is seen as clear plaques in the otherwise confluent bacterial growth The highest serum dilution which gives growth inhibition indicates the titre of vibriocidal antibodies in the tested serum The method has a reproducibility within one titre step and is more sensitive than conventional methods for evaluating such antibodies

A modification was also introduced allowing exclusion of the serial diluting procedure From circular basins made in the inoculated agar 6 µl of test sera together with a standard serum with known content of vibriocidal antibodies are left for 24 hours prediffusion at + 4°C Thereafter the plate is flooded with complement and incubated at 37°C for 4 hours The zone areas of the clear plaques are directly proportional to the content of vibriocidal antibodies in the serum. By comparison with the standard serum the vibriocidal antibody contents of the test sera can be calculated

Acta path microbiol scand Section B 79 449–458 1971

ZONAL CENTRIFUGATION OF SOLUBLE IMMUNE COMPLEXES

2 Analysis of Antigen Antibody Mixtures at Equilibrium over Various Parts of the Precipitin Curve

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In an attempt to determine the distribution of soluble complexes formed when ^{131}I labelled HSA reacts with fluorescein labelled anti HSA we have used zonal centrifugation in a sucrose gradient combined with a subsequent analysis of the cuts by radioactivity, fluorescence and polarization of fluorescence measurements The method is both reproducible and sensitive and has a potential advantage over other methods for separating soluble complexes in that the sedimentation coefficients as well as antibody ratio of the complexes can be determined on microgram quantities of sample

Using this method we have been able to follow the dissociation of antigen antibody complexes with time and to demonstrate the building up of large numbers of complexes at the equivalence zone Full technical details of the method are given together with suggestions for further improvements

There has been much interest in the soluble complexes formed between antigens and antibodies In the clinic immune complexes have been studied in connection with diseases, such as glomerulonephritis and lupus erythematosus (4) and in addition experimental studies have been carried out on the role of immune complexes in allergic reactions (7, 10)

From a physico chemical point of view the mechanism of formation of antigen antibody complexes is of interest especially since the development of Palmiter & Aladjem's theory for the precipitin reaction (12) This theory is of particular interest when attempting an analysis of immunodiffusion (2, 8) and in obtaining a deeper understanding of the precipitin reaction in general

In the previous publication we have given details of a zonal centrifugation system (14) which allows an analysis of the HSA rabbit anti HSA system to be made In this paper we present a more detailed analysis of five points on the precipitin curve, from antigen excess to moderate antibody excess, by zonal centrifugation followed by analysis of the cuts by radioactive counting, fluorescence and polarization of fluorescence measurements We subsequently hope to make a complete

Received 19 i 71

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identification of the individual complexes based on $S_{20,w}$ and $D_{20,w}$ measurements for each complex Zonal centrifugation has the potential of giving more information than has been possible by other methods such as analytical ultracentrifugation (13), sucrose gradient centrifugation (9), polyacrylamide electrophoresis (6) or gel filtration on Sephadex G200 (3), not least because it is both an analytical and a preparative method

MATERIALS AND METHODS

(i) *Antigen* The antigen used was a monomer preparation of HSA, prepared by alkylation of ^{131}I-HSA with iodoacetamide followed by gel filtration

750 µl of ^{131}I-HSA (Amersham) was diluted with an equal volume of a pH 8 1 Tris buffer (1 M with respect to NaCl, 0 01 M with respect to Tris), the mixture cooled to 4° C, and 1500 µl of 0 005 M iodoacetamide in pH 8 1 Tris added After 1½ hours the monomer was separated by gel filtration on a K 25/100 Sephadex G 100 column, equilibrated in pH 8 3 borate buffer The second (major) peak was pooled and concentrated through a Diaflo UM 10 membrane (cut-off at m wt 10 000), and subsequently dialysed against pH 8 0 Tris buffer, 0 05 M To attain a good separation of the monomer from the polymeric forms of HSA, it was found to be essential to use borate buffer (Tris buffer was quite unsuited)

The time-course of alkylation was assessed as follows 6 ml of 2 per cent HSA was diluted with 6 ml of Tris pH 8 1 buffer, and alkylated as before with 12 ml of 0 005 M iodoacetamide At time intervals of 5 10, 15 30, 45, 60 and 120 minutes, 2 ml samples were taken and the free protein separated from iodoacetamide on 20 cm G 25 columns, equilibrated with borate buffer The flow of the sample was assessed by placing 100 µl of Blue Dextran 2000 on the columns just prior to sample application The free protein was collected, protein concentration determined by the O D at 280 nm (after having applied a correction factor for any traces of Blue Dextran which has absorption maxima at 280 nm and 620 nm) 5 µl of DTNB (5,5-Dithiobis2-nitrobenzoic Acid, 20 mg/ml in acetone*) was added to 3 ml of protein solution, and the extinction at 420 nm read after exactly 15 minutes These experiments showed that all the SH groups were alkylated within 90 minutes, and in fact the reaction was approximately 90 per cent complete after 45 minutes

* Ellmans reagent *Ellman, G L* Arch Biochem, Biophys *82* 70 77, 1959

The homogeneity of the alkylated monomer was assessed by acrylamide disc electrophoresis The non-alkylated HSA showed multiple bands, while the alkylated product showed a single band

(ii) *Antibody*. Antibody (rabbit anti HSA, Dakopatts, Copenhagen) was labelled with FITC isomer I (Sigma) as follows·

5 ml (53 mg) of anti HSA solution, previously dialysed against pH 9 0 bicarbonate buffer, 0 5 M, was mixed with 4 mg of fluorescein isothiocyanate (FITC), isomer I (Sigma), and the reaction allowed to proceed under magnetic stirring for 18 hours at 4° C. The pH was adjusted to 9 0 in the initial stages of the reaction

The labelled antibody was separated from the free fluorescein by gel filtration on a K 25/100 Sephadex G 25 column, the column being equilibrated and eluted with pH 8 3 borate buffer The fractions corresponding to the latter half of the asymmetric protein peak were pooled, and concentrated through a Diaflo UM 10 membrane to a final protein concentration of 0 85 mg/ml The sample was finally dialysed against the pH 8 0 0 05 M Tris buffer used in the zonal centrifugation

The FITC immunoglobulin ratio was calculated by means of the formula

$$\frac{(\mathrm{FITC})}{(\mathrm{Ab})} = \frac{(E_{494} - 0.5\,E_{280})}{(E_{280} - 0.30\,E_{494})} \times \frac{\varepsilon_{494}\,\mathrm{FITC}}{\varepsilon_{280}\,\mathrm{Ab}}$$

where ε_{494}FITC isomer I, at pH 8 0 at 22° C = 7 71 × 10^4 and ε_{280}Ab under the same condition = 2 08 × 10^5 The molar dye to protein ratio for the FITC-Ab used in these experiments was 2 45

(iii) *Titration of FITC-Ab* was carried out by the antigen serial dilution method The tubes were incubated at 37° C for 1 hour and left overnight in the cold room The washed precipitates were dissolved in IN NaOH and both the extinction at 280 nm and the fluorescence intensity at 484 519 nm measured

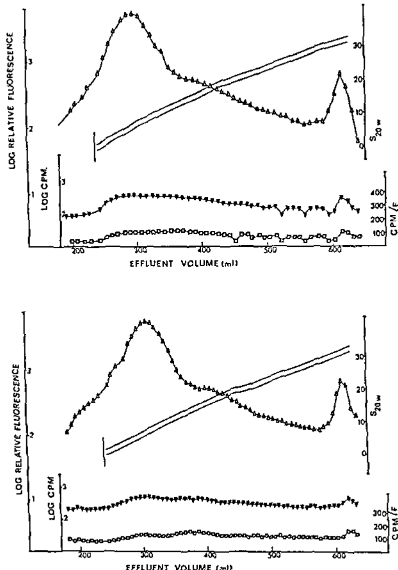
(iv) *^{131}I-HSA protein estimation* was carried out using *Laurell's rocket method* (11)

(v) *Preparation of soluble complexes* as described previously (14

(vi) *Zonal centrifugation* was carried out with an isokinetic sucrose gradient in a B XIV Aluminium rotor as described previously

(vii) *Analysis of the zonal centrifugation cuts* Each cut was counted in a Packard 3003 Tri-Carb Scintillation Spectrometer with a 2" crystal, and subsequently both fluorescence and polarisation of fluorescence measured within five days using an Aminco-Bowman spectrophotofluorometer The spectrophotofluorometer was equipped with a 150 watt Hanovia Xenon lamp an ellipsoid mirror focusing system and a solid state Harke direct photometer photomultiplier Since polarization measurements were made at the same time as the

Fig 1 A and B Reproducibility of the method 68 mg of ^{131}I HSA monomer were added to 850 µg FITC anti HSA, and incubated 1 hour at 37° C, then 4° C overnight The sample was then applied to the feed line of a B XIV Aluminium rotor, and run for 306 minutes at 30425 rpm (temperature 10 5° C) The other experimental conditions are as described in the text Polarization of fluorescence data for the experiment 1 B are shown in Fig 2 The signatures are as follows Δ log relative fluorescence, ▼ log CPM, □ CPM/F, and the full line S20, W values



fluorescence measurements, it proved convenient to take the fluorescence as the sum of $I_{\parallel}$ and $I_{\perp}$ ($I_{\parallel}$ represents the intensity of fluorescence when both the excitation and emission polarizers are set to pass vertically polarized light and $I_{\perp}$ the intensity of fluorescence measured with the excitation polarizer set to pass vertically polarized light and the emission polarizer set to pass horizontally polarized light) A standard solution of FITC isomer I (2 0 µg/ml) was measured with each set of experiments to check source intensity and lamp stability All measurements were carried out at 12° C with 18 nm band width excitation and emission slits (*Excitation was at 484 nm, Emission at 519 nm*) The 484 nm wavelength was chosen to avoid spurious results due to light scatter The polarized fluorescence P was measured as

$$P = \frac{I_{\parallel} - I_{\perp} G}{I_{\parallel} + I_{\perp} G}$$

(The grating factor G was 0 83) The cuvette faces were kept moisture free by blowing a stream of cold, freeze-dried air over the faces The usual precautions were taken to eliminate interference to the measurements by dust particles (scatter) Measurements were not made until temperature equilibrium had been attained in the sample The temperature of the cuvette contents was measured

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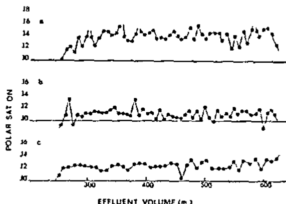


Fig 2 Changes in the polarization of fluorescence curve for experiment 1 B with time *a* after 5 days *b* after 8 days, *c* after 20 days Polarization of fluorescence (●) was measured as described in the text (Points occuring either side of an obvious maximum or minimum, which cannot be defined precisely, have not been linked with a line)

using a Mettler platinum thermistor with digital read out to 0 1° C Cooling the cuvette was achieved by circulating 70 per cent ethanol at —15° C through the cuvette housing No attempt was made at this stage to correct the polarization of fluorescence for differences in the viscosity of the solution in the various cuts

RESULTS

A *Reproducibility of the Method*

In Figs 1 A and 1 B are shown the results of two runs in the antibody excess region made at an interval of three days using the same quantities and concentrations of antigen and antibody i e 20 μl ^{131}I HSA + 1000 μl Ab FITC (68 μg ^{131}I-HSA and 850 μg Ab FITC) The curves for radioactive antigen, fluorescent antibody and ratio of antigen/antibody coincide extremely well However the rather complex polarization of fluorescence curves which are not included in these figures for the sake of clarity, show variations with time as will be discussed below When repeating polarization of fluorescence measurements within a few hours the values obtained are identical to within 0 003 polarization units and the fluorescence intensity to under 1 per cent

B *Stability of the Immune Complexes*

Heideman, (6) using polyacrylamide electrophoresis, was able to demonstrate the relative stability of immune complexes by acrylamide electrophoresis since an individual band representing a single complex gave only a single band on elution and re-electrophoresis In our experiments shown in Fig 2, we have re-analyzed a single run (that shown in Fig 1 A) at time intervals of 5 8 and 20 days These experiments show that in contrast to *Heideman*'s observations the immune complexes are undergoing a gradual break-down to smaller units This is indicated both by the broadening of the polarization of fluorescence peaks with time and by the fact that the value of the polarization of fluorescence falls from a maximum of about 0 15 at 5 days to about 0 13 after 20 days Unfortunately, transportation problems have prevented an immediate analysis of the zonal cuts but it is obvious that in future work this will have to be done

C *Analysis of the Complexes at Various Points on the Precipitin Curve*

Having shown the reproducibility of the method, we ran several experiments corresponding to various Ag Ab ratios starting at large antigen excess and proceeding to moderate antibody excess In these experiments 850 μg Ab-FITC was added to 300 180 144 96 and 48 μg of ^{131}I HSA monomer, respectively These results are shown in Figs 3 A to 3 E There are several points of interest in these curves

The Radioactivity Curve

Three and in some cases four distinct peaks are seen in *all* the experiments corresponding to

a) the free antigen peak about 4 65 S)
b) the AgAb and Ag Ab and Ag_2Ab_3 complexes
c) insoluble complexes sedimentation coefficient > 30 S

These peaks do not show up so well in the figures presented here, where radioactivity has been plotted on a logarithmic scale, in order to allow a direct comparison to be made between the various experiments As we progress from antigen excess to antibody excess, the radioactivity curve becomes broader and in linear plots shows increasingly greater detail, which is in agreement with *Palmiter & Aladjem*s description of the antigen antibody reaction—as p (the fraction of antigen sites reacted) increases, the distribution of complexes with respect to the molecular weight becomes wider up to the point of equivalence, and then decreases The distribution of radioactivity is greatest in experiments 3 C and 3 D, representing points just on the antigen excess side of equivalence Conversely in antigen excess, small complexes, presumably AgAb and Ag_2Ab_1, predominate Other well known properties of the precipitin curve which are demonstrated in these experiments are firstly the predominance of free antigen, and smaller complexes in antigen excess (Experiments 3 A, 3 B and 3 C), and secondly, the increase in the amount of bound antigen in the form of insoluble complexes which occurs on going from antigen excess to equivalence

The Fluorescent Antibody Curve

The fluorescence curve does not show as much detail as the radioactive antigen one until antibody excess is reached The first shoulder or peak occurring fairly early on in all the experiments, corresponds to an $S_{20,w}$ value of 6 3 to 7 3 and represents free antibody

The shoulder or peaks following the main peak, representing the antibody component of the soluble complexes, becomes broader as one proceeds along the precipitin curve from antibody excess to antigen excess, so that in

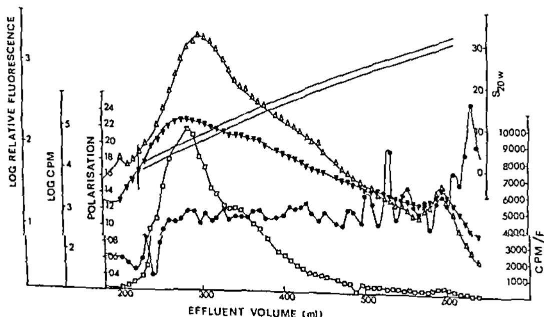


Fig 3 A

Fig 3 A to E Separation of soluble complexes under the same experimental conditions as described in the legend to Fig 1 The amount of antigen and antibody mixed are as follows A 300 μg, B 180 μg C 144 μg D 96 μg and E 48 μg of ^{131}I HSA monomer, respectively, were added to 850 μg Ab-FITC The meaning of the signatures is explained in the legend to Fig 1 and 2

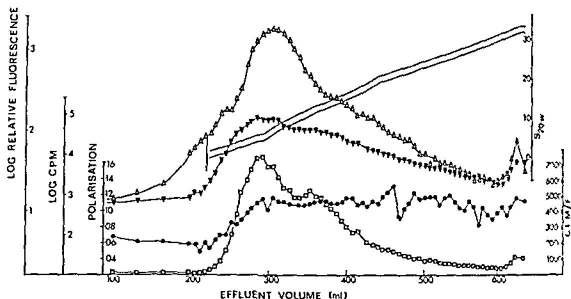


Fig 3 B

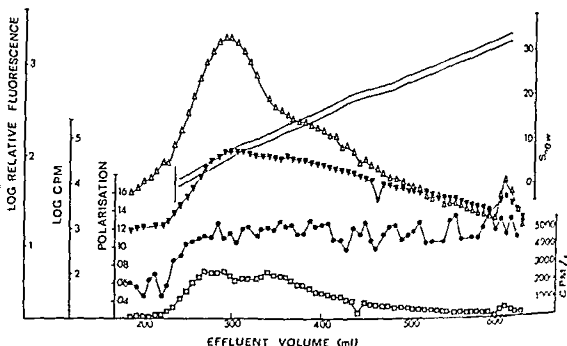


Fig 3 C

antigen excess the antibody exists in the form of complexes mainly of the soluble type

The Ag/Ab curve (Radioactivity/fluorescence intensity—(cmp/F))

This curve is characterized by two clear maxima which show a very characteristic alteration on proceeding from antigen to antibody excess The first peak around 4 5 S is most dominant in antigen excess and of course marks the free antigen component b t

EFFLUENT VOLUME (ml)

Fig 3 D

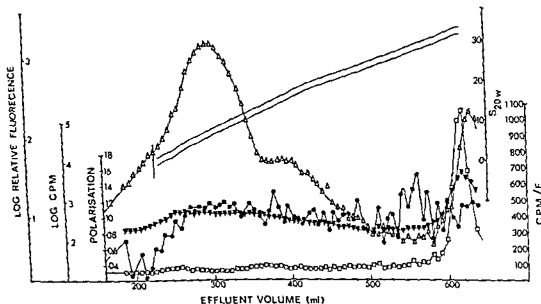


Fig 3 E

progressively decreases in relation to the second peak as one approaches the antibody excess zone Slightly to the antigen excess side of equivalence the peaks are almost equal, and in antibody excess, the latter peak is the predominant one. The interpretation of this curve is complicated by the fact that complexes of differing composition, but having approximately the same $S_{20,w}$ (*e g* $AgAb_2$ m wt 369 000 and Ag_2Ab_2 m wt 438 000)

will have differing Ag Ab ratios, and conversely that the same Ag Ab ratio does not define the complex (*e g* Ag4Ab and Ag_2Ab_2) However, the second peak having a sedimentation coefficient of about 11 S, could represent the complex Ag_2Ab (9)

The Polarization of Fluorescence Curve

The polarization of fluorescence curves are, in spite of the reservations made earlier, interesting, and show a complicated spectrum of soluble complexes The polarized fluorescence curve tends to increase with the sedimentation coefficient of the complex, the various peaks which occur presumably marking the mass centres of the individual complexes However, it is surprising that the values of the polarization are much lower than those attained in experiments in which the *antigen* is labelled with fluorescein, and the antibody is "native" With fluorescein on the HSA, polarization values of up to 0 35 have been obtained in our laboratory (unpublished results) or something like the double of the present values We are currently investigating the effect on the zonal centrifugation experiments of attaching this label to the antigen rather than to the antibody

That the peaks in the polarization curve are significant is indicated by the fact that in many cases shoulders in the antigen and antibody curves in fact coincide with maxima in the polarization of fluorescence curve Also, the polarization curve is reproducible to a high degree, as indicated by repeated measurements on individual cuts Furthermore, the relative complexity of the radioactive antigen and antibody curves at equivalence is paralleled by a corresponding complexity in the polarization of fluorescence curve In antigen (and antibody) excess where the distributions of complexes theoretically should be the narrowest, there is indeed a notably more simple polarization of fluorescence curve

D *Antibody Alone*

In the experiment shown in Fig 4 fluorescein labelled anti-HSA has been run alone under the same experimental conditions as for the complexes (N B the free antigen curve has been shown in a previous publication (14)) There are two clear peaks corresponding to IgG, and in addition a smaller amount of aggregated material There are only minor variations in the polarization of fluorescence curve which have maxima at the 7 and 19 S points in the elution volume These are followed by a few peaks which are reproducible on repeated measurements but

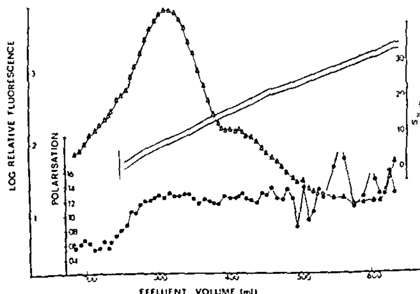


Fig 4 Antibody ... 850 μg Ab-FITC alone E... perimental conditions ... signatures are as described in the text to Figs 1 and ...

whose nature is unknown A certain amount of the antibody seems to exist in an insoluble form as demonstrated by the small peak at the edge of the rotor

DISCUSSION

In both *Goldberg*s (5) and *Palmiter & Aladjem*s treatment (12), given the number of reacting antigen and antibody molecules their valencies and information as to the rate constants for the forward and reverse reactions one can predict the theoretical distribution of the complexes at any given time most usually at equilibrium

As yet no absolute proof of the validity of *Palmiter & Aladjem*s theory has been obtained *Aladjem et al* (1) in a preliminary study compared their theory with experimental results, and showed that in their system consisting of egg albumin as antigen and a water soluble antibody globulin fraction of rabbit antiserum as antibody the theory correctly predicted the experimentally observed supernatant composition in antibody excess equivalence and antigen excess They emphasize that the results published in this article are only approximate and suggested that for a complete proof of their theory precipitin data using purified antibody were needed together with weight average molecular weight measurements of the supernatants so that the molecular weight and concentration of each type of complex in various regions of the precipitin curve could also be made

Thus in order to test the validity of these theoretical treatments it is therefore necessary to analyse a given antigen antibody reaction mixture at equilibrium to see what species and in what numbers they exist Experimentally zonal centrifugation seemed to offer certain advantages in that it is rapid and analysis and recovery of the fractions presents no problem in contrast to for example polyacrylamide electrophoresis In addition the zonal rotor may be used analytically so that sedimentation coefficients may be determined Conventional analytical ultracentrifugation while giving more exact sedimentation coefficients does not give the same possibilities for subsequent recovery and analysis of small fractions

The results presented here have partially fulfilled the requirements, although the resolution is not as complete as could be wished, and the sedimentation coefficients could not be determined closer than ± 0 5 Accurate determination of the sedimentation coefficients is a necessary prerequisite for the identification of the various species since from the sedimentation constant and the diffusion coefficient it is possible to make estimates of the molecular weights The molecular weight in combination with a double labelling technique as applied here and polarization of fluorescence measurements should allow an unequivocal identification of the species to be made The error in the determination of $S_{20\ W}$ stems from two main sources

a) the difficulty in running experiments at a well defined temperature (the viscosity of the medium amongst other factors determines the sedimentation rate of a particle in a centrifugal field) and
b) occasional small errors due to leakage during loading and unloading combined with other volume errors We hope to eliminate both these errors shortly by means of improved experimental set up including the incorporation of protein markers with known sedimentation coefficient

The second area in which the present results could be improved is by carrying out the polarization of fluorescence measurements as soon as possible after the centrifugation before the complexes have had time to dissociate appreciably The results obtained by measurements of polarized fluorescence at different times following zonal centrifugation indicate that a slow but significant dissociation of the isolated complexes occurs as would in fact be expected This is in contrast to *Heideman*s observations as to the stability

of immune complexes run on acrylamide gels, where a band corresponding to a particular complex could be eluted and re-run as a single band

We are at present continuing these zonal centrifugation experiments in order to improve the resolution of the method (15) Our aim is to be able to resolve and characterize all the soluble (and insoluble) immune complexes and by this means determine whether the distribution of species as determined by *Palmiter & Aladjem*'s theory is in fact the same as that found experimentally

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We wish to thank Mrs *Lene Moe*, *Kirsten H[illegible]gard* and *Kirsten Briem* for excellent technical assistance, Mr *Rud Larsen* for assistance with the centrifuge, and Miss *Benedikte Hedegaard Petersen* for running the acrylamide gel electrophoresis experiments

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Acta path microbiol scand Section B 79, 459–465, 1971

PREPARATION OF ANTISERA TO BOVINE IMMUNOGLOBULIN CLASSES BY IMMUNIZATION WITH AGAR-GEL PRECIPITATES

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Antisera to bovine IgM and IgA were developed by immunizing rabbits with specific agar gel immune precipitates By precipitation with a polyvalent anti immunoglobulin serum the immunoglobulin classes were isolated from solutions which contained IgM (G 200 gel filtration fractions) or IgA (lacrimal fluid) as the only or at least predominant immunoglobulin Antibodies developed against shared antigenic determinants (light polypeptide chains) and non immunoglobulin proteins were easily removed using selected absorptive materials Attempts to produce antisera exclusively reactive with either IgG slow or IgG fast were unsuccessful presumably due to slight antigenicity of subclass-specific determinants

Heterogeneity of immunoglobulins has been clearly evidenced by immunochemical analyses in agar-gels Antigenic determinants define the immunochemical heterogeneity and grouping of immunoglobulins into classes and subclasses is based upon the recognition of these characteristics

In cattle there exist at least three classes of immunoglobulins IgG, IgM and IgA The IgG which comprise two subclasses IgG slow, IgG fast, has been characterized in detail by *Murphy et al* (1965) *Pierce & Feinstein* (1965) *Aalund* (1968) and *Kickhöfen et al* (1968) Also IgM has been described in fairly great detail (*Murphy et al* 1965), and recently IgA has been recognized in various fluids such as colostrum, saliva, bile, tears and serum (*Mach et al* 1969, *Vaerman* 1970, *Pedersen & Nansen* 1971) In lacrimal fluid IgA is a major component (*Pedersen Nansen* 1971)

Specific antisera against IgM (*Klaus & Jones* 1969), IgA (*Mach et al* 1969) and IgG subclasses (*Vaerman* 1970, *Nansen* 1970) have been developed by immunization of rabbits with isolated, chromatographic fractions and subsequent absorption of antibodies produced against shared immunoglobulin determinants (light polypeptide chains) Isolation of normal immunoglobulins, in particular IgM and IgA is often a difficult and tedious task involving chromatography and/or salting-out procedures It has been demonstrated, however, that antisera can be easily developed against single diffusible antigenic substances by immunizing heterologous animal species with specific

Received 8 1 71

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of immune complexes run on acrylamide gels, where a band corresponding to a particular complex could be eluted and re-run as a single band

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Acta path microbiol scand Section B 79, 459-465, 1971

PREPARATION OF ANTISERA TO BOVINE IMMUNOGLOBULIN CLASSES BY IMMUNIZATION WITH AGAR-GEL PRECIPITATES

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Antisera to bovine IgM and IgA were developed by immunizing rabbits with specific agar gel immune precipitates By precipitation with a polyvalent anti immunoglobulin serum the immunoglobulin classes were isolated from solutions which contained IgM (G 200 gel filtration fractions) or IgA (lacrimal fluid) as the only or at least predominant immuno globulin Antibodies developed against shared antigenic determinants (light polypeptide chains) and non immunoglobulin proteins were easily removed using selected absorptive materials Attempts to produce antisera exclusively reactive with either IgG slow or IgG fast were unsuccessful presumably due to slight antigenicity of subclass-specific determinants

Heterogeneity of immunoglobulins has been clearly evidenced by immunochemical analyses in agar gels Antigenic determinants define the immunochemical heterogeneity and grouping of immunoglobulins into classes and subclasses is based upon the recognition of these characteristics

In cattle there exist at least three classes of immunoglobulins IgG, IgM and IgA The IgG, which comprise two subclasses IgG slow IgG fast, has been characterized in detail by *Murphy et al* (1965) *Pierce & Feinstein* (1965) *Aalund* (1968) and *Kickhöfen et al* (1968) Also IgM has been described in fairly great detail (*Murphy et al* 1965), and recently IgA has been recognized in various fluids such as colostrum, saliva, bile, tears and serum (*Mach et al* 1969, *Vaerman* 1970, *Pedersen & Nansen* 1971) In lacrimal fluid IgA is a major component (*Pedersen Nansen* 1971)

Specific antisera against IgM (*Klaus & Jones* 1969), IgA (*Mach et al* 1969) and IgG subclasses (*Vaerman* 1970, *Nansen* 1970) have been developed by immunization of rabbits with isolated, chromatographic fractions and subsequent absorption of antibodies produced against shared immunoglobulin determinants (light polypeptide chains) Isolation of normal immunoglobulins, in particular IgM and IgA, is often a difficult and tedious task involving chromatography and/or salting-out procedures It has been demonstrated, however, that antisera can be easily developed against single diffusible antigenic substances by immunizing heterologous animal species with specific

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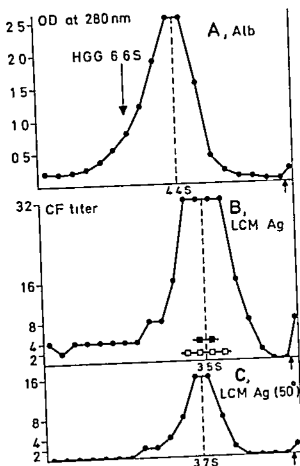


Fig 4 Investigation of LCM virus specific antigen by rate zonal centrifugation 0 3 ml samples were layered on 4 6 ml of 5 20 per cent sucrose gradients A 1 5 per cent bovine plasma albumin B sucrose acetone extracted LCM antigen C same antigen exposed to 50° C for 30 min Middle of sample layers indicated by short arrows Centrifugation was performed with a SW 50L rotor for 16 hours at 40 000 rpm 3° C In addition to CF titration antigen in the fractions from gradient B was studied by immunodiffusion—from each fraction material was transferred directly or after incubation at 50° C for 30 min to neighbouring wells Strong precipitin lines of thermolabile and thermostable antigen were found as indicated by white and black squares respectively Long arrow (gradient A) shows relative position of 6 6 S human gammaglobulin to bovine plasma albumin as found in corresponding runs

for 30 min was used in the following to inactivate thermolabile antigen At this temperature the precipitin band corresponding to thermostable antigen was not affected, and with different preparations 1/8–1/4 of the CF activity remained

Rate zonal centrifugation Linear 5 20 per cent sucrose gradients of 4 6 ml each were used to take advantage of a nearly linear relationship between distance travelled from meniscus and sedimentation constant (8) For approximate determination of sedimentation constants comparisons were made within the same run, with reference materials of known S values The validity of the technique was confirmed by runs in which bovine plasma albumin (Calbiochem A grade) 4 4 S and human gammaglobulin (Calbiochem, A grade) 6 6 S were compared (Fig 4 A indicates where the 6 6 S peak of gammaglobulin was found relative to the position of albumin)

Fig 4 shows the results from a run (40 000 rpm for 16 hours at 3° C), in which bovine plasma albumin (A), LCM antigen (B) and LCM antigen heated to 50° C for 30 min (C) were layered on the gradients described The antigen preparation used was purified by ammonium sulfate precipitation and CF titer was 256 The position of albumin was recognized by OD at 280 nm (A) In the other gradients the distribution of absorbing material was quite even (not shown)

In the sample used for gradient B the presence of both antigens was demonstrated It is apparent, however that specific antigen (determined by CF activity) was accumulated in a single and fairly well defined peak which has clearly entered the gradient In the lower regions a small but significant amount of antigen was present From the distances between peaks and the middle of the sample layers (A and B) a sedimentation constant, 3 5 S was calculated for the complementfixing material

CF titer of the sample used for gradient C was 64, and only thermostable antigen was present It is apparent that a peak of CF activity was found in a position cl to the peak recognized f r the unheated , paration This finding suggested that the sedimentation constants for the two antigen were very similar, and this was confirmed when fractions obtained from gradient

were studied by immunodiffusion. Samples from each fraction were applied untreated and after 30 min at 50° C. Clearcut precipitin lines were localized as indicated by the squares in Fig 4 B. It can be seen that both lines were distinct corresponding to the peak of CF activity.

As far as the thermolabile antigen apparently was of protein nature (see below), a crude estimation of molecular weight (MW) may be obtained from the following equation (8) valid for proteins $S_1/S_2 = (MW_1/MW_2)^{2/3}$. With a MW of 67,000 for bovine plasma albumin 3.5 S will correspond to about 48 000.

Attempts to further characterization of the antigens. The antigens were assumed to be of protein nature. In order to establish this treatment with proteolytic enzymes was carried out.

Antigen with an admixture of trypsin (4 mg/ml) was incubated at 37° C and pH 7.4 for varying periods. After incubation soya bean trypsin inhibitor was added (8 mg/ml) and the mixture cooled on an ice bath. To unveil any irrelevant destruction of antigen during the procedure and interference in the gel precipitation, antigen alone was incubated for the same period at 37° C, whereupon trypsin and trypsin inhibitor were added simultaneously in quantities as above. As may be seen from Fig 5, both precipitin lines were unaffected corresponding to the controls. The action of trypsin during the incubation was however, clear. After 5 min one of the antigens was already obviously reduced, and after 30 min it was absent (see Fig 5). The other antigen, however, was completely unaffected even when incubation was extended to 4 hours. Reaction of identity was demonstrated between the thermostable and the trypsin resistant antigens.

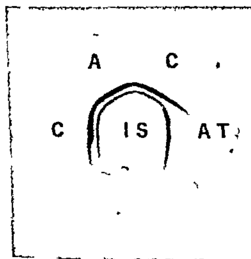


Fig 5 Antigen remaining after exposure to proteolytic activity A sucrose acetone extracted antigen AT antigen incubated with trypsin (4 mg/ml at 37° and pH 7.4 for 30 min followed by addition of soya bean trypsin inhibitor (8 mg/ml) and cooling on ice bath C controls incubated for the same period after which trypsin and inhibitor have been added simultaneously IS immune rabbit serum

Trypsin resistant proteins are not unique, as the substrate specificity is somewhat restricted, with hydrolysis only of peptidebonds adjacent to carboxyl groups of arginine and lysine. More remarkable was a similar resistance of the thermostable antigen to prolonged incubation with *Streptomyces griseus* protease. This enzyme is exceptional among proteinases in that it hydrolyses almost all peptidebonds until the majority of amino acids are liberated as free amino acids (9). Sucrose acetone extracted antigen was incubated for 24 hours with 2 mg *Streptomyces griseus* protease per ml, tp 37° C and pH 7.4. Gel diffusion was performed at 2° C with the appearance, as above, of one unaffected precipitin line corresponding to the thermostable antigen. The same experiment was also made using the splenic antigen which gave rise to only one precipitin line (Fig 2). As may be seen from Fig 6, the presence of both antigens in this material was clearly demonstrated and identity between thermostable and protease resistant antigens was established. Enzymatic function was checked by similar incubation of antigen preparations to which bovine serum albumin or ovalbumin had been added. Amounts (2 mg/ml) which far exceeded the amount of virus specific antigen were completely destroyed as indicated by immunodiffusion with specific antisera.

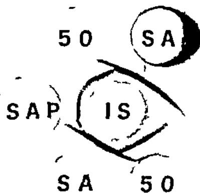


Fig 6 Analysis of antigen from spleens of infected mice SA splenic antigen SAP splenic antigen incubated with *Streptomyces griseus protease* (2 mg/ml) at 37° C for 24 hours 50 splenic antigen heated to 50° C for 30 min IS immune rabbit serum

Attempts to examine the possibility that antigen or hapten of non protein nature could be involved were made As LCM virus belongs to the ether sensitive viruses it is likely that material of lipid character is included in the virion However both the antigens described proved to resist extraction with diethylether Antigen in Tris NaCl buffer (or dried) was added to 20 vol diethylether and incubated at 0° C for 30 min with intermittent shaking Neither thermostable nor thermolabile antigen was extracted or inactivated by this treatment

Methods for chemical characterization of antigen after precipitation in gels are described by *Uriel* (16) Following these directions antigen giving rise to two distinct precipitin lines was studied Glycoproteins and polysaccharides were searched for by the periodic acid NADI reaction with negative result Staining procedure involving pyronin Y for characterization of nucleoprotein or nucleic acid was also negative Finally as expected Oil red O and Sudan III revealed no lipid associated with the precipitates

DISCUSSION

By far the greater part of the antigen demonstrated by complement fixation and immunodiffusion was localized in the cells In culture fluid no antigen was detected by direct testing despite the fact that much higher titers of infectious virus were present here than in the cellular preparations These relations, as well as the separation of antigen from sedimentating virus during centrifugations emphasize that virions contribute very little if anything to the activity of antigens described in the present paper As indicated the results were apparently not due to any artificial breakdown of intact virus particles during the operations rather antigenic material of low S value exist preformed in the cells Probably the widespread cellular immunofluorescence (> 95 per cent positive cells) is also an expression of such antigenic material rather than of infectious virus The findings agree with electron microscopic observations by *Dalton et al* (1) on LCM virus infected cultures The virus particles were primarily of extracellular location and apparently very few intact cells contained virus However, as much as 90 per cent of the cells were stained with fluorescent antiserum Discrepancies between antigen demonstrated by immunofluorescence and infectious virus were also noticed by *Lehmann Grube et al* (5) in studies on serial cultures of LCM infected cells In some passages no trace of infectious virus was found nevertheless 90 per cent of the cells were positive by staining

While sucrose acetone extraction was found superior in respect to the quantitative yield there was apparently no qualitative difference when the different preparations of antigen were studied by immunodiffusion or differential centrifugation By immunodiffusion experiments the presence of two virus specific antigens was demonstrated and both were classified as s [illegible] antigens One antigen was apparently a thermolabile protein which was inactivated at temperatures above 40° C The other one the thermostable antigen was found to resist proteolytic treat

ment Even incubation with the most potent protease, *Streptomyces griseus* protease (9), for 24 hours under optimal conditions did not break down the antigen The nature of this antigen was not established by the experiments performed It was always associated with the weaker band in gel precipitation and seemed to be responsible for a minor part of the total CF activity

Sedimentation in rate zonal experiments gave rise to only one peak of CF activity corresponding to about 3 5 S Immunodiffusion findings demonstrated the presence of both antigens in the peak fractions and suggested that they had very similar sedimentation constants Calculations, valid for estimation of protein molecular weights, were applied to the thermolabile protein antigen and indicated a molecular weight about 48,000 This moderate size, as well as the apparent absence of antigen in upper fractions corresponding to lower S values make it probable that the peak antigen is composed of single antigen molecules whereas antigen present in the lower regions probably stems from aggregation characteristic of virus proteins However, reservation must be made with respect to the thermostable antigen insofar as its nature was not elucidated

There is no experimental evidence that infectious LCM virus in any way participates in the virus specific complement fixation immunoprecipitation or immunofluorescence reactions Even the modest CF activity observed after concentration of culture fluid seems to be exponent for the smaller soluble' antigens This phenomenon could be a question of quantities only However there are clues suggestive of some qualitative antigenic dissimilarity between 'soluble antigen and the virion *Smadel* (15) using soluble antigen prepared from spleens was able to carry out absorption of all CF antibodies in immune guinea pig serum without reducing its capacity to neutralize infectious virus *Volkert et al* (18) and *Lundstedt* (6) have shown that in mice viremia and humoral immunity —high titers of infectious virus and even very high CF antibody titers—may coexist for several months, apparently without interference

ADDENDUM

Preliminary immunodiffusion studies on LCM antigen have recently been described by *M Simon* (Acta virol *14* 369–376, 1970) and *C Chastel* (Acta virol *14* 507–509, 1970) In agreement with the present findings different preparations of CF antigen gave rise to two major precipitin lines However, a total of five or four antigens are described

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Acta path microbiol scand Section B 79, 475–482 1971

EFFECT OF STRONG AND WEAK HISTOCOMPATIBILITY ANTIGENS ON THE MIXED LYMPHOCYTE CULTURE REACTION IN RATS

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Individual rats belonging to two different F2 crosses were serotyped for H1 (AgB) determined antigens and tested in mixed lymphocyte culture (MLC) against parental strain rats *In agreement with the general contention that MLC reactivity is controlled solely by* the major histocompatibility locus of the species, all H1 different mixtures were found to stimulate, while H1 identical mixtures failed to stimulate The lack of MLC stimulation by minor loci antigens may be due to low frequency of antigen sensitive cells against these antigens Immunization may be expected to increase their frequency and indeed in some combinations low but clear cut stimulation was produced by non H1 antigens after specific immunization. In the H1 different AS AS2 combination an unexpected discrepancy was found between the relative antigenic strength as measured by MLC on one side and by the local graft versus host assay (12, 13) and by kidney graft survival on the other side Possible reasons for this discrepancy are discussed

Reactivity in mixed lymphocyte culture (MLC) seems generally to be controlled by antigens determined by the major histocompatibility locus Extensive studies in man have demonstrated a close correlation between HL-A determined antigens and the results of MLC (2, 21, 25) Only a few exceptions have been reported and the significance of these is not clear at present (29) In the rat reactivity in MLC has likewise been shown to depend on disparity with respect to H1 (AgB) determined antigens (4, 7, 22) In mice, however, several investigators have now reported stimulation in MLC between H2 identical cell donors (1, 9, 10, 15, 17, 26) In one study the stimulation was explained by the additive effect of multiple minor loci differences (17), whereas in another study the stimulating capacity was found to segregate as a single unit the possible linkage of which to the H2 locus has not been determined as yet (10)

In the present study MLC was carried out between parental strain and F2 generation rats and the results confirmed the previous demonstration of strict H1 control of MLC reactivity The lack of MLC stimulation by minor loci antigens may be due to low frequency of antigen sensitive cells against these antigens However, immunization against minor loci antigens may be expected to in-

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crease the frequency of these cells to a level which could lead to demonstrable MLC stimulation Experiments were carried out to investigate this possibility and evidence of weak MLC stimulation after immunization against minor loci antigens was obtained in some combinations

MATERIAL AND METHODS

Cell donors Inbred rats of the Fischer (F), Brown Norway (BN), AS and AS2 strains and various hybrids raised from these were used as cell donors The F and BN rats were obtained from Dr *H O Sjögren*, Lund Sweden, and the AS and AS2 rats from Dr *N W Nisbet*, Oswestry, Shropshire, England, and maintained here as inbred strains The BN and LEW BN rats were obtained from Drs *O Stark* and *V Kren*, Prague, Czechoslovakia The LEW BN strain is congenic with the LEW strain and carries the H1 allele of BN

Method of culturing leucocytes MEM S (Eagle) tissue culture medium (Flow Laboratories) added glutamine 2 mM, pyruvate 1 mM and benzylpenicillin natrium 150 I U per ml supplemented with 10 per cent fresh rat serum was used as culture medium. Serum was obtained from (F × BN) F1 or (AS × BN) F1 hybrids The blood was allowed to clot at room temperature for 30 minutes and subsequently kept at 4°C for 3–4 hours before the serum was collected

Cells were obtained from heart blood drawn in plastic syringes From parental and F1 rats blood could be pooled from several syngeneic animals whereas only blood from individual F2 animals was used Adult rats usually survived the withdrawal of 4–5 ml

The blood was defibrinated for 10 minutes in Erlenmeyer flasks containing glass beads and subsequently mixed with 1 5 vol of a mixture of 4 parts of MEM S and 1 part of 20 per cent dextran (m wt 248 000 Pharmachem Corporation U S A) in 0 9 per cent saline The red cells were sedimented for 1 2 hours at 37°C after which the supernatants containing the leucocytes were collected These were centrifuged (170 G 10 minutes) and the cells resuspended in culture medium at a concentration of 3 × 10^6 mononuclear cells per ml

In some experiments supernatants containing cells to be used as target cells were added Mitomycin C (Sigma) 25 µg per ml and incubated for 30 minutes at 37°C (3) Subsequently the cells were spun down washed twice in culture medium (170 G 10 minutes) and finally resuspended in culture medium at a concentration of 3 × 10^6 mononuclear cells per ml

Mixed cultures were prepared from equal volumes of responder and target cells and unstimulated controls consisted of unmixed cell suspensions of either kind In experiments where the target cells were mitomycin treated unstimulated controls were prepared from equal volumes of responder cells and autologous mitomycin treated cells As controls for the efficiency of the mitomycin treatment, mixtures of equal volumes of mitomycin treated cells from two H1 different donors were employed

Culture vials were flat bottomed glass tubes (6/H/1001 Johnson & Jorgensen (Trident) Ltd London) with loosely fitted screw caps containing 0 5 ml cell suspension The cultures were maintained at 37°C in a humified atmosphere of 10 per cent O_2, 4 per cent CO_2 and 86 per cent N_2 (9)

The medium was changed after two days by removing 300 µl of medium with a Carlsberg pipette without disturbing the cells and replacing it by an equal volume of fresh medium stored at 4°C and prewarmed to 37°C

All cultures were harvested in triplicate or in quadruplicate Approximately 72 hours after preparation of the cultures 0 1 µCi ^{14}C thymidine (sp a 56 mCi/mM The Radiochemical Centre Amersham England) was added per culture and 16 hours later the cells were processed for liquid scintillation counting as previously described (23 24) Quenching was assessed by the external standard method and was found in accordance with previous findings, to be constant irrespective of the number of cells harvested and the activity in the culture Consequently all results are expressed as mean counts per minute per culture

When deviations from the procedure described were employed these are indicated in the result section

Serotyping Haemagglutinating antisera specific for the respective H1 antigens of the parental strains were used in typing of the F2 hybrids

Most antisera were obtained by reciprocal immunizations of the AS AS2 and BN F strains by skin grafting followed at weekly intervals by i p injections of liver and spleen cells However in accordance with *Elces* findings (8) no AS2 anti AS serum was obtained by means of the immunization schedule described A weak AS2 anti AS serum was nevertheless found in an AS2 rat with an AS heart graft The latter was used for typing in the dilution 1 20 whereas the other sera were diluted 1 100 in TBS

The erythrocyte suspensions were approx 2 per cent made up in 9 parts of Plasmagel and 1 part of normal F1 serum Two drops of antiserum dilution and one drop of erythrocyte suspension were incubated at 37°C for 2 hours in small plastic tubes before macroscopical reading

Grafting Approximately 15 15 mm sq and

of abdominal full thickness skin were grafted to the dorsum of the thorax Rejection was scored as the day on which the whole graft surface was converted to a scab Kidney grafting was performed with minor modifications as described by *Sakai et al* (18 19) Heart grafting was performed essentially as described by *Pettirossi et al* (16)

RESULTS

Table 1 shows the results of MLC between 24 (AS × AS2) F2 hybrids and the two parental strains AS and AS2 The H1 genotypes of the individual F2 animals were determined in advance by means of haemagglutination In each experiment cells from an F2 hybrid of each of the three possible H1 genotypes (AS2/AS2 AS/AS2, and AS/AS), were mitomycin treated and mixed with cells from each of the two parental strains As seen from the table the heterozygotes always stimulated both parental strains, while the homozygotes only stimulated the parental strain carrying the opposite H1 allele

Furthermore the results allow a comparison to be made within each experiment between the stimulation of AS parental cells by AS2 H1 antigens and of AS2 parental cells by AS H1 antigens The AS2 cells gave regularly a higher response than the AS cells In order further to substantiate this finding 4 additional experiments were carried out where cells from individual AS and AS2 rats were stimulated by the same (AS × AS2) F1 cells (without mitomycin treatment) The culture media were in these experiments supplemented with (AS × AS2) F1 serum As seen from Table 2 the AS2 cells were also here regularly stimulated to a higher level than the AS cells

These results may be compared with the results of organ grafting between the AS and AS2 strains as shown in Table 3 The expectation that AS2 recipients would reject the graft from the opposite strain at a faster rate than would AS recipients is obviously not borne out Admittedly the variation obtained with heart grafts is so great in either group so as to make it doubtful whether any real difference exists On the other hand in kidney survival times there is no overlapping and the difference goes in the opposite direction to what MLC would have predicted This difference in kidney graft survival is in agreement with previous findings of *Salaman*

TABLE 1 *CPM in MLC between AS AS2 and (AS × AS2) F2 Rats*

Exp No	Responding cells	H1 genotype of (AS × AS2) F2 target cells AS2/AS2	AS/AS2	AS/AS	Unstimulated controls	Mitomycin controls
1	AS	2258	1567	269	100	353
	AS2	200	3105	7426	73	
2	AS	2779	1454	192	183	–
	AS2	130	4072	4895	201	
3	AS	1747	703	191	226	239
	AS2	76	1076	3117	94	
4	AS	5321	4196	341	153	894
	AS2	230	5828	8049	195	
5	AS	8292	2233	161	313	756
	AS2	221	2418	5440	250	
6	AS	5370	3233	164	208	148
	AS2	133	2598	3434	94	
7	AS	3349	2160	144	88	363
	AS2	141	3753	5453	122	
8	AS	4737	2230	136	89	1049
	AS2	215	2699	–	97	

In each experiment mitomycin treated cells from three (AS × AS2) F2 hybrids were used to stimulate cells from the two parental strains AS and AS2

Unstimulated controls consisted of mixtures of untreated parental cells and autologous mitomycin treated cells

Mitomycin controls consisted of mixtures of H1 non identical mitomycin treated cells

TABLE 2 *CPM in MLC between AS AS2 and (AS × AS2) F1 Rats*

Exp No	1	2	3	4
AS + (AS × AS2)	1944	2334	1675	1517
AS2 + (AS × AS2)	3205	3309	4139	3793

Unmixed controls of AS AS2 and (AS × AS2) F1 <200 CPM

In each experiment cells from individual AS and AS2 rats were stimulated by the same (AS × AS2) F1 cells

TABLE 3 *Kidney and Heart Transplantation from AS2→AS and AS→AS2*

	Survival times in days	
	Kidney	Heart
AS2→AS	10	5†
	10	41
	12	225§
	10	8
	10	7
	9	7
AS→AS2	69	10
	14	15
	94§	20
	50*	45
	24†	140*
	94§	9

* still functioning
† death from non immunological reasons
§ killed with the graft still functioning
In the group AS2→AS the heart and kidney grafts were taken from the same donor and transplanted into the recipients whereas in the group AS→AS2 the two grafts were taken from different donors

(20), in the same strain combinations The relative strength of these two combinations as determined in this laboratory by the local lymph node enlargement assay of GVH reaction (12 13) suggests, however, the same ranking order as does the actual outcome of kidney grafting

Table 4 shows the results of MLC between 39 (F × BN) F2 hybrids and the parental F strain The F2 hybrids were again genotyped in advance by serotyping and in each experiment cells from one (Exp No 1-3) or two (Exp No 4–8) hybrids of each type were mitomycin treated and mixed with cells from the F strain As seen from the Table only hybrid cells carrying the BN H1 allele stimulated the F cells

In either strain combination tested no indication of stimulation due to non H1 determined antigens was obtained the mean ratio in H1 identical combinations between mixtures and controls being 1 14±0 64 (range 0 29–2 74) As previously pointed out (4) the

TABLE 4 *CPM in MLC between F and (F × BN) F2 Rats*

Exp No	H1 genotype of (F × BN) F2 target cells BN/BN	BN/F	F/F	Unstimulated controls	Mitomycin controls
1	4391	3179	62	87	381
2	7254	6408	99	134	697
3	6669	2898	161	120	167
4	3484	2527	122	95	577
	2666	1958	114		
5	18570	7227	238	294	337
	11082	5505	340		
6	36240	24144	156	542	263
	28151	16806	220		
7	5529	2325	152	8	334
	3378	2704	128		
8	9928	7771	264	234	229
	8998	5141	162		

Experiments 1 3 contain three and experiments 4 8 conta x F BN) F2 hybrids the cells of which were mitomycin treated and used t stimulate cells from the parental F strain
Unstimulated controls consisted of mixtures of untreated F cells a d mitomycin treated F cells
Mitomycin controls consisted of mixtures of H1 non identical mitom n treated cells

AS–AS2 combination was not the best choice for the investigation of a possible effect of multiple minor loci differences because these two strains have been shown to differ at only 4–5 histocompatibility loci one of which is the H1 locus (14) Skin grafts transplanted from the (AS × AS2) F2 hybrids of the AS/AS genotype to AS recipients showed a wide range of survival times from 14 days to possibly permanent survival (14, 14, 21, 30, 38, 50, >250, >250 days)

The F and BN strains, however, differed at sufficient minor histocompatibility loci to ensure rather fast rejection of H1 identical F2 hybrid skin grafts in F strain hosts In all cases rejection was complete between 14 and 18 days Yet no sign of reactivity in MLC between these F2 hybrids and the F strain was obtained

The rather wide range found in the ratios between mixtures and controls in H1 identical combinations might conceivably be due to minimal stimulation in some of these mixtures However, neither in the AS–AS2 nor in the F–BN combination was any correlation found between skin graft survival and MLC results in the H1 identical pairs of hybrid and parental rats

In an attempt to boost MLC reactivity against minor loci antigens by means of immunization, eight (F × BN) F2 hybrids of the F/BN H1 genotype were transplanted with two consecutive skin grafts from (F × BN) F1 donors (14 days interval) The F1 hybrids supposedly possess all minor histocompatibility antigens of the two parental strains, whereas the F2 hybrids will on average be homozygous at half of the corresponding loci F1 hybrids will therefore be incompatible for individual F2 hybrids with respect to a variable but unknown, number of minor loci antigens against which sensitization may occur

All second set skin grafts were rejected after 11 days except in one animal (F2, No 35) which rejected the skin graft after 9 days The results of the MLCs performed appear in Table 5 In seven out of eight animals did the sensitization by skin grafting lead to increased MLC reactivity but only F2, No 35 showed significant stimulation, the ratio between mixtures and controls being higher than the mean ratio plus four standard deviations as calculated from the results before grafting

TABLE 5 *MLC of Cells from F1 Hybrids and H1 Heterozygous F2 between the Fischer and BN Strains The F2 Hybrids are Tested before and after Sensitization with F1 Skin Grafts*

F2 No	Before grafting	14 days after first graft	14 days after second graft
27	0 67	1,70	1,81
29	1,45	1 80	2 44
30	2,03	1,30	1,76
35	0,78	3 80	3,10
39	1 17	2 04	2 66
42	1 05	1 55	1 54
46	0,88	1 73	1,30
51	0 68	1,52	1,56
Mean 1 08 ± 0 46 (s d)			

The figures represent the ratio between CPM of the F2 + F1 mixture and mean of the unmixed controls

Mixtures of F + (F × BN) F1 were employed as positive controls, these exceeded in all cases 13 times the mean control values

The BN and LEW strains have been found to differ with respect to 14–16 histocompatibility loci (6) and the BN and LEW BN strains may therefore be suitable for the investigation of a possible effect in MLC of multiple minor loci incompatibilities Two pairs of BN and LEW BN animals were cross immunized with two skin grafts (14 days interval) followed by 3 weekly injections of spleen and lymph node cells in the foot pads and in the angles of the mouth Each animal received a total of 80×10^6 leucocytes at each immunization All second set skin grafts were rejected after 8 days in both directions One month after the last immunization two way MLC was performed between the members of each of the pairs together with 3 pairs of non immunized controls As a positive control cells from an AS2

TABLE 3 *Kidney and Heart Transplantation from AS2→AS and AS→AS2*

	Survival times in days	
	Kidney	Heart
AS2→AS	10	5†
	10	41
	12	225§
	10	8
	10	7
	9	7
AS→AS2	69	10
	14	15
	94§	20
	50*	45
	24†	140*
	94§	9

* still functioning
† death from non immunological reasons
§ killed with the graft still functioning
In the group AS2→AS the heart and kidney grafts were taken from the same donor and transplanted into the recipients whereas in the group AS→AS2 the two grafts were taken from different donors

(20), in the same strain combinations The relative strength of these two combinations as determined in this laboratory by the local lymph node enlargement assay of GVH reaction (12 13) suggests, however the same ranking order as does the actual outcome of kidney grafting

Table 4 shows the results of MLC between 39 (F × BN) F2 hybrids and the parental F strain The F2 hybrids were again genotyped in advance by serotyping and in each experiment cells from one (Exp No 1-3) or two (Exp No 4-8) hybrids of each type were mitomycin treated and mixed with cells from the F strain As seen from the Table only hybrid cells carrying the BN H1 allele stimulated the F cells

In either strain combination tested no indication of stimulation due to non H1 determined antigens was obtained the mean ratio in H1 identical combinations between mixtures and controls being 1 14±0 64 (range 0 29–2 74) As previously pointed out (4) the

TABLE 4 *CPM in MLC between F and (F × BN) F2 Rats*

Exp No	H1 genotype of (F × BN) F2 target cells BN/BN	BN/F	F/F	Unstimulated controls	Mitomycin controls
1	4391	3179	62	87	381
2	7254	6408	99	134	627
3	6669	2898	161	120	167
4	3484 2666	2527 1958	122 114	95	577
5	18570 11082	7227 5505	238 340	294	337
6	36240 28151	24144 16806	156 220	542	263
7	5529 3378	2325 2704	152 128	282	334
8	9928 8998	7771 5141	264 162	239	229

Experiments 1 3 contain three and experiments 4 8 contain six (F×BN) F2 hybrids the cells of which were mitomycin treated and used to stimulate cells from the parental F strain
Unstimulated controls consisted of mixtures of untreated F cells and mitomycin treated F cells
Mitomycin controls consisted of mixtures of H1 non identical mitomycin treated cells

Unfortunately the investigation of the LEW BN with BN combination was restricted by the limited number of animals available, and the results obtained must therefore be regarded as preliminary. However, the immunized pairs showed a clear stimulation, although very low compared with the mixtures of H1 disparity included in the same experiment. However, comparison of the levels of stimulation between immunized and non immunized cells may be obscured by earlier occurrence of target cell destruction in immunized as compared to non immunized mixtures. Further studies are obviously needed before the effect of pre immunization in MLC may be assessed.

Elves has studied the strength of MLC stimulation in both directions between AS and AS2, and he found stronger stimulation of AS2 by AS than in the opposite direction when the blastic response was measured after 6 days, whereas at 4 days the opposite result was found (7). *Festenstein* finds in agreement with the present data that AS2 is stimulated stronger by AS antigens than is AS by AS2 antigens (11).

The AS with AS2 combination represents an unsuspected and unexplained discrepancy between MLC and GVH estimates of the relative antigenic strength. A further apparent contrast is the fact that MLC gives significant stimulation with non immune cells only in combinations of H1 disparity. The GVH reaction, although largely determined by H1, can in fact be demonstrated also in H1 identical combinations, albeit with much higher cell doses. One could therefore say that the MLC reaction as presently performed is less sensitive than is the GVH reaction to weak antigenic differences. Presumably this is a matter of culture technique. There is little reason to suggest that the two reactions are not in principle measuring the same thing, i.e. primary immune reactions caused predominantly by histocompatibility antigens of the strong locus of the species.

No convincing evidence for rejection of organ grafts due to incompatibility for minor loci antigens alone has so far been obtained in rats (4, 5). The possibility still remains, however, that minor loci antigens in conjunction with H1 determined antigens may influence organ graft survival. If this is true the discrepancy found between results of MLC and organ graft survival may again be explained by the lack of sensitivity in MLC towards minor loci antigens. However, modifying factors on graft rejection such as the production of enhancing antibodies may as well explain the discrepancy found. The ability to produce enhancing antibodies may be more pronounced in some donor host combinations than in others, and this ability may be independent of the H1 determined antigenic strength as measured by MLC. Further studies are obviously needed before the predictive value of MLC in organ grafting may be assessed.

Close inspection of Tables 1 and 4 will show a striking effect which has so far received no mention. That is the fact that parental cells are regularly stimulated more by F2 hybrid target cells which are homozygous for the H1 allele of the opposite parent than they are by heterozygous cells. This suggestion of a marked gene dose effect has been investigated in more detail and will form the subject of the accompanying report.

We wish to thank Dr *Hilliard Festenstein* for helpful discussions and *Merete Tjalve* for excellent technical assistance.

This work was supported by the *Danish Medical Research Council*.

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Acta path microbiol scand Section B 79, 483–488, 1971

EVIDENCE FOR GENE DOSE EFFECT OF H1 (AgB) DETERMINED ANTIGENS IN RAT MIXED LYMPHOCYTE CULTURE

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The stimulation obtained in mixed lymphocyte cultures (MLC) with mitomycin treated target cells of a different H1 (AgB) type has been investigated for the possible effect of gene dosage Almost invariably it was found that target cells carrying two rather than one chromosome with a particular foreign H1 allele caused a significantly higher degree of stimulation This fact pertained to the comparison of target cells from homozygous pure strain and the F1 hybrid It also holds for the comparison of F2 hybrids homozygous and heterozygous respectively for a given foreign H1 allele (3) The greater degree of stimulation by homozygous target cells was manifested during the entire culture period and was found to exist over a wide range of target cell concentrations These findings contrast with other published data when no evidence for a gene dose effect of H1 determined antigens was found The possible reasons for this discrepancy are discussed

A close correlation between H1 (Ag-B) determined antigens and the responses in mixed lymphocyte culture (MLC) has been demonstrated (for references, see (3)) Target cells, being different from the responding cells with respect to H1 determined antigens, always stimulate, whereas H1 identical mixtures never show stimulation Furthermore, studies of two F2 generations have shown that cell donors homozygous for the foreign H1 alleles always stimulated parental strain cells to a greater level than heterozygous cell donors, thus suggesting a gene dose effect of H1 determined antigens in MLC (3) This finding is of importance for the interpretation of the additive effect recently demonstrated for H1 determined antigens in MLC (5), and additional studies were therefore undertaken in order to elucidate this point

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MATERIAL AND METHODS

The rat strains used and the detailed method of culturing lymphocytes was described in a previous report (3) Culture media consisted of MEM-S (Eagle) added glutamine, pyruvate and penicillin, supplemented with 10 per cent fresh rat serum

The target cells were incubated with Mitomycin C, 25 μg per ml at 37° C for 30 minutes and subsequently washed twice in culture medium

The cells were cultured in 0 5 ml volumes in flat bottomed glass tubes with loosely fitted screw caps and maintained at 37° C in a humified atmosphere of 10 per cent O_2, 4 per cent CO_2 and 86 per cent N_2 Unless otherwise stated, each culture contained 0 75 × 10^6 responding and 0 75 × 10^6 target lymphocytes

Change of medium was performed at day two and day four after preparation of the cultures

The cellular proliferation was assessed by pulse

labelling with ^{14}C thymidine Approximately 72 hours after preparation of the cultures 0 1 μCi ^{14}C-thymidine (sp a. 56 mCi/mM) was added per culture, and 16 hours later the cells were processed for liquid scintillation counting When the response was measured at successive days of culture, only 4 hours incubation with ^{14}C thymidine was employed

All cultures were harvested in triplicate and the results expressed as mean counts per minute per culture

RESULTS

Since mitomycin treatment of the target cells was employed in the present study, experiments were carried out to assess the influence of this treatment on the stimulatory capacity of the cells For genetic reasons only the parental cells will be stimulated in a parent —F1 mixture (4) and any alteration in the response brought about by mitomycin treatment of the F1 cells must therefore reflect the effect of this treatment on the stimulatory capacity of the F1 cell suspension Figure 1 and Table 1 show that the mitomycin treatment caused slightly lower responses when measured early in the culture period whereas the subsequent cell proliferation was not inhibited by the mitomycin treatment Consequently, mitomycin treatment of the target cells was considered suitable for rat MLC

TABLE 1 *Effect of Mitomycin Treatment on the Stimulatory Capacity of F1 Hybrid Cells in MLC*

Exp no	Mitomycin treatment of (AS × BN) F1 cells	Mean cpm per culture day 3	day 4	day 5
1			1566	2757
	+		1251	3056
2			1035	2182
	+		998	3910
3		479	1331	2096
	+	496	1194	1825
4		437	1212	3807
	+	353	1084	3162
5	—	823	2745	4284
	+	546	1812	4323
6		359	1221	2863
	+	275	889	3052

In each experiment AS cells were stimulated with untreated and mitomycin treated (AS × BN) F1 cells

Unmixed controls of AS (AS × BN) and (AS × BN) m < 200 cpm m indicates mitomycin treated cells

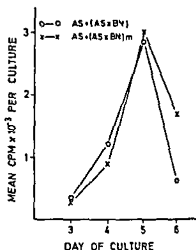


Fig 1 This figure shows experiment No 6 Table 1 Cells from AS rats were stimulated with untreated and mitomycin treated (AS × BN) F1 cells The responses were measured from day 3 to day 6 of culture m indicates mitomycin treated cells

In order to investigate the dose response relationship on stimulation with homozygous as well as heterozygous target cells 6 experiments were performed where a constant number of responding cells were stimulated with increasing numbers of target cells Fig 2 shows the results Usually maximal stimulation was obtained when the ratio between target and responding cells was 2 1 and higher numbers of target cells always caused inhibition of the response However, in some experiments levelling off or even inhibition of the response was seen when the ratio exceeded 1 1, and this held true both for stimulation with homozygous and heterozygous target cells The 1 1 ratio was therefore chosen for the following experiments

In the previously mentioned studies (3 cells from F2 hybrids being homozyg

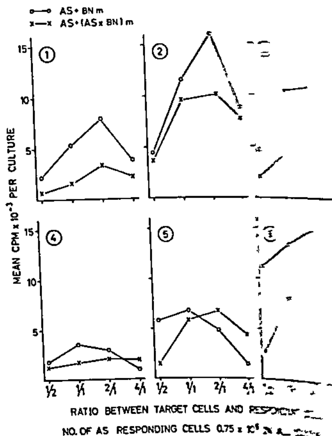


Fig 2 'm' indicates mitomycin treated

with respect to the foreign H1 alleles always stimulated parental cells to a higher degree than heterozygous F2 cells However control mixtures containing H1 different mitomycin treated cells often showed stimulation to a level slightly above that of the unstimulated controls indicating that the mitomycin treatment did not prevent all target cells from dividing The possibility therefore existed that the additional activity found in cultures stimulated with homozygous target cells was due to cell division of these cells whereas the heterozygous target cells for genetic reasons could not be stimulated

Consequently experiments were performed (Table 2 A) where AS cells were stimulated by mitomycin treated BN and (AS × BN) F1 cells (AS ... cells were ... AS and (AS ... BN + (AS ... efficiency of ... tures of ... were ... clearly higher ... compared ... obtained ... AS cells ... mitomycin ... periments ... ceeded the ... BN + BN ... the activity ... not alone ...

which ... either ... intra- ... ani- ... ation ... trans- ... hout ... divi- ... ation ... s et ... seen ... intra- ... cor- ... cyte ... tres, ... ume ... thus, ... these ... the ... The ... ion ... nti ... ion ... nti ... ity ... nt ... h ... e ... or ... u ... tro ... el ... nd ... body ... 1046 ... rela ... mune ... rma ... Clin ... A

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EFFECT OF MITOMYCIN TREATMENT ON THE STIMULATORY CAPACITY OF F_1 HYBRID CELLS IN MLC

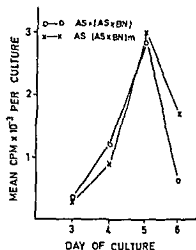


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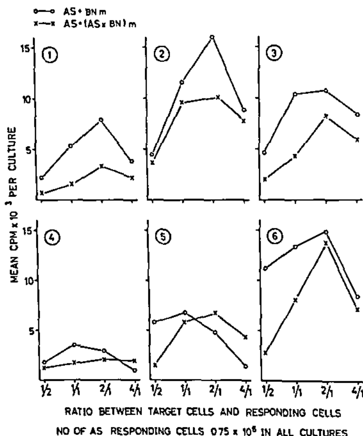


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Consequently experiments were performed Table 2 A) where AS cells were stimulated by mitomycin treated BN and (AS × BN) F1 cells (AS + BNm AS + (AS × BN)m) and BN cells were stimulated by mitomycin treated AS and (AS × BN) F1 cells (BN + ASm, BN + (AS × BN)m) As controls for the efficiency of the mitomycin treatment, mix tures of mitomycin treated AS and BN cells were employed (ASm + BNm) Again clearly higher stimulation by homozygous as compared to heterozygous target cells was obtained (except in the stimulation of the AS cells in experiments 2 and 6), and the mitomycin controls in some experiments (ex periments 1 and 8) again *significantly* ex ceeded the unstimulated controls (AS + ASm BN + BNm) However in quantitative term the activity in the mitomyc not alone account for the

TABLE 2 A *CPM in MLC Between AS, BN, and (AS × BN) F1 Rats*

Exp No	1	2	3	4	5	6	7	8	9
AS + BNm	7361	4267	6340	6244	19007	5193	3159	15139	154[illegible]
AS + (AS × BN)m	5731	4190	2385	3601	10053	5419	1831	9683	8[illegible]
Difference	1630	77	3955	2643	8954	44	1328	5454	61[illegible]
BN + ASm	3605	1771	1609	1086	2976	1276	1741	3315	3[illegible]
BN + (AS × BN)m	1279	483	254	384	753	886	662	2804	15[illegible]
Difference	2326	1288	1355	702	2223	390	1079	1041	1[illegible]
ASm + BNm	735	402	116	187	206	305	172	796	5[illegible]

AS + ASm or BN + BNm Mean of 18 cultures 189 ± 101 (s d)
m indicates mitomycin treated cells

TABLE 2 B *CPM in MLC Between AS and (AS × BN) F1 Rats*

Mixtures and No of Cells × 10⁵ per Culture	Exp No			
AS + ASm + (AS × BN) m	6	7	8	9
3 75 + 0 + 7 50	1105	945	4232	2663
3 75 + 3 75 + 7,50	1096	1267	4135	2399

m indicates mitomycin treated cells

tween stimulation by homozygous and heterozygous target cells

Such a quantitative comparison could be questioned on the ground that the conditions for proliferation of mitomycin treated cells might be better in a culture where a great number of non treated cells are proliferating as compared to a mitomycin control where only a few cells are dividing This possibility, however may be excluded for the following reason If proliferation of BNm cells in the mixtures AS + BNm were to explain the excess activity in these cultures as compared to the AS + (AS × BN)m mixtures then this difference should never exceed the activity found in the simultaneous BN + ASm mixtures where no attempt was made to inhibit proliferation of the BN cells As seen from the table, the difference in 5 out of 9 experiments clearly exceeded the activity found in the BN + ASm mixtures, and proliferation of BNm cells alone could therefore not account for the differences found

Furthermore, in experiments 6-9 AS + (AS × BN)m mixtures were prepared containing only half the usually employed number of responding AS cells together with similar mixtures containing in addition an equal number of ASm cells (AS + ASm + (AS × BN)m) The results are presented in Table 2 B In 3 out of 4 experiments the presence of mitomycin treated AS cells did not increase the culture response whereas mixtures containing the usual number of AS cells (7 5 × 10⁵) showed clearly greater activity (Table 2 A)

The possibility existed that initially in the cultures an equal number of cells were triggered off on stimulation with homozygous and heterozygous target cells and that the difference seen when measuring the response later might be due to a more rapid cell proliferation in cultures stimulated with homozygous as compared to heterozygous target cells Fig 3 shows two experiments representative of [illegible] the nine experiment

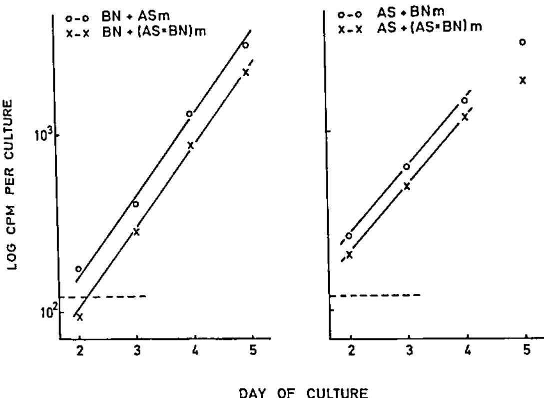


Fig 3 Dotted lines indicates level of unstimulated controls m indicates mitomycin treated cells

performed demonstrating that the gene dose effect was discernible from the time of significant activity in the cultures and that during the exponential growth phase the same slope was observed on stimulation with homozygous and heterozygous target cells thus suggesting equal generation times of the responding cells on stimulation with both kinds of target cells

DISCUSSION

The results obtained in the present study confirm the previous finding of a gene dose effect of H1 determined antigens in MLC (3) Figs 2 and 3 show that the gene dose effect could be demonstrated over a wide range of mitomycin treated target cell concentrations and during the entire culture period

These results contrast with the data of *Wilson* & *Nowell* (5) who found no difference in the response on stimulation with homozygous and heterozygous target cells in MLC The reason for this discrepancy is not clear at present *Wilson* & *Nowell* only measured the dose response relationship when stimulating with heterozygous F1 cells, and it is possible that the optimal concentration found for heterozygous F1 cells and subsequently employed in the gene dose experiments was inhibitory for the homozygous

target cells In the culture system described in this report, levelling off or inhibition of the response was seen more often in the case of homozygous as compared to heterozygous target cells when the ratio between target cells and responding cells exceeded 1 1

Another explanation may be that Wilson & Nowell in order to produce one way reactions on stimulation with homozygous target cells did not use mitomycin treatment but used instead cell donors rendered tolerant to the antigens of the responding cells by neonatal injection of bone marrow cells of the responder genotype Thus their target cell donors could have been high level chimaeras Consequently, the real number of stimulating homozygous target cells may have been lower than estimated and the lower cell number compensated for unknowingly by the gene dose effect

As a third possibility remains that the tolerant chimaeric state may result in the production by donor cells of blocking antibodies directed against the host antigens (1) which in turn may inhibit the stimulatory capacity of the host cells in MLC

Mitomycin treatment was in the present study found to cause only a slight decrease in the stimulatory capacity of the target cells However both kinds of target cells were mitomycin treated and if this treatment was responsible for the demonstrated gene dose effect it would have to be postulated that the heterozygous cells are more vulnerable to this treatment than are homozygous cells This possibility seems very unlikely but the experiments should perhaps be repeated employing X ray treatment of the target cells

In contrast to the heterozygous target cells the homozygous target cells were exposed to alien histocompatibility antigens carried by the responding cells and it was therefore necessary to consider the possible effects of this recognition in the cultures Table 2 A and 2 B show that the additional activity found in cultures stimulated by homozygous cells cannot be due solely, if at all to proliferation of the mitomycin treated cells themselves However it may be imagined that mitomycin treated cells at contact with alien histocompatibility antigens could elaborate mitogenic or potentiating factors which unspecifically increased the response of the untreated cells in the cultures (2) This possibility has not been critically excluded in the present series of experiments but the results presented in Table 2 B gives no indication of such activity in the cultures

Wilson & Nowell (5) demonstrated an additive effect of H1 determined antigens in MLC and because of the lack of gene dose effect in their control experiments the additive effect was taken as evidence for unipotentiality of antigen sensitive cells of specificity for H1 determined antigens This conclusion may in fact prove to be correct but the alternative possibility that the antigen sensitive cell may be multipotential cannot as yet be ruled out

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Merete Tjalve is thanked for excellent technical assistance

This work was supported by the *Danish Medical Research Council*

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Acta path microbiol scand Section B 79, 489–494, 1971

ANTIGEN-INDUCED LYMPHOCYTE TRANSFORMATION *IN VITRO* DURING PRIMARY IMMUNIZATION IN MAN

1 *Development and Course*

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and SOREN FREIESLEBEN SORENSEN

Departments of Medicine A and P,
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Fifteen patients were immunized with killed *brucella abortus Bang* The *in vitro* transformation response of blood lymphocytes to antigen was followed for 15 days after the immunization Five patients, all undergoing treatment with immunosuppressive/anti inflammatory drugs, showed little or no response In some of the responding patients, transformation was observed as early as 3 days after immunization Early occurrence of transformation was associated with high rates of thymidine incorporation at response maximum During the development of the response, the lymphocytes became more sensitive to low antigen concentrations

Antigen-induced lymphocyte transformation has been extensively employed as an *in vitro* parameter of lymphocyte reactivity (*Ling* 1968) However, studies in man of lymphocyte transformation during development of the immune response following primary immunization are rare The present paper reports a study of the development of transformation induced by killed brucella bacteria following immunization with this micro-organism In the following paper (*Soborg et al* 1971), the transformation response is compared to antigen induced leucocyte migration inhibition and to the appearance of circulating antibodies

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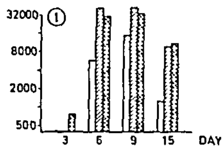
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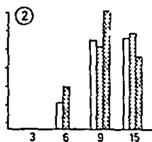
Fifteen adult in patients with different medical disorders were examined (Table 1) The criterion of selection was that they showed no evidence of previous exposure to brucella as judged by a negative leucocyte migration test and absence of agglutinins in serum (*Soborg et al* 1971) Before immunization, the purpose and the procedures were explained to the patients, and their consent obtained

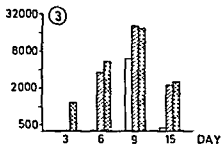
Immunization was performed by the injection of a suspension of killed *brucella abortus Bang* (Statens Seruminstitut), containing 10^9 bacteria per ml The patients were given 0 3 ml subcutaneously + 0 1 ml intracutaneously in one thigh, 0 3 ml subcutaneously in the other Blood samples were drawn on days 3 6, 9, and 15 after immunization

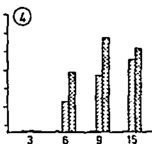
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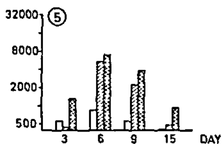
For lymphocyte culture, blood was defibrinated and sedimented with dextran (*Sorensen et al* 1969) The supernatant was cultured in aliquots of 4 ml, it contained approximately 10^6 lymphocytes and 10 neutrophils per ml, and 33 per cent of the

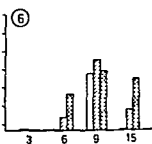

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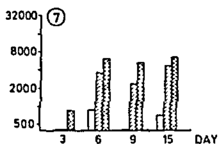

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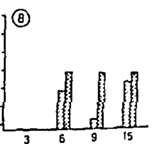

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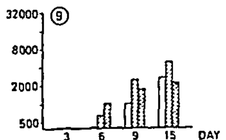

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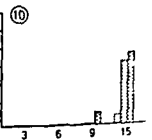

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tion of the lymphocyte transformation However *Hinz et al* (1970) studied a patient who showed a positive skin test to tuberculin but whose lymphocytes failed to transform *in vitro* during prednisone treatment, in this case the failure to respond was apparently a property of the lymphocytes since substitution of normal serum for the patient's serum in the cell cultures was not associated with greater stimulation

The remaining 10 patients all showed antigen induced lymphocyte transformation following immunization Early occurrence of lymphocyte transformation with maximal response on day 6 or 9 and declining reactivity on day 15 was associated with high rates of thymidine incorporation indicating participation of larger numbers of lymphocytes in the response Low thymidine incorporation rates were found in patients showing late occurrence of transformation and still increasing reactivity 15 days after the immunization

Studies of lymphocyte transformation following vaccination of human subjects with smallpox (*Sarkany* & *Caron* 1966) and BCG (*Sarkany* & *Hales* 1968) have shown considerably longer latency periods 2 to 3 weeks before reactivity occurred However primary responses to inoculation of human subjects with keyhole limpet haemocyanin (*Curtis et al* 1970) and alligator erythrocytes (*Harris et al* 1970) occurred approximately 1 week after immunization From the present study it appears that lymphocyte transformation occurs early after vaccination with brucella in 4 out of 10 responding patients already 3 days after vaccination

Fig 3 Development of lymphocyte reactivity as judged by ^{14}C thymidine incorporation after immunization of the donors on day 0 The cultures were set up on the day indicated Ordinate counts per minute per 10^6 lymphocytes at initiation of culture

☐ 0.25×10^8 brucella/ml culture
2 5
× 12 5

Sarkany & *Hales* (1968) found a temporary fall in the degree of lymphocyte transformation approximately 4 weeks after BCG vaccination followed by a secondary peak It is possible therefore that the decline seen on day 15 in most patients in the present study represents the initiation of a temporary fall

Early after immunization responses were best obtained with the highest antigen concentration while the cells eventually became more sensitive to lower antigen concentrations This observation is in accordance with *Curtis et al*'s (1970) finding of a strong increase in the responses obtained with increasing antigen concentrations 7 days after immunization while this increase levelled off during the following 50 days These findings are most likely explained by the production of cells with receptors of increasing affinity during the course of immunization

The evalution of lymphocyte transformation after immunization as compared to inhibition of leucocyte migration and to antibody production will be discussed in the following article

We wish to thank Miss *Mette Schibbye* for competent technical assistance

Supported by *Statens Almindelige Videnskabsfond* Grant No L 136/68

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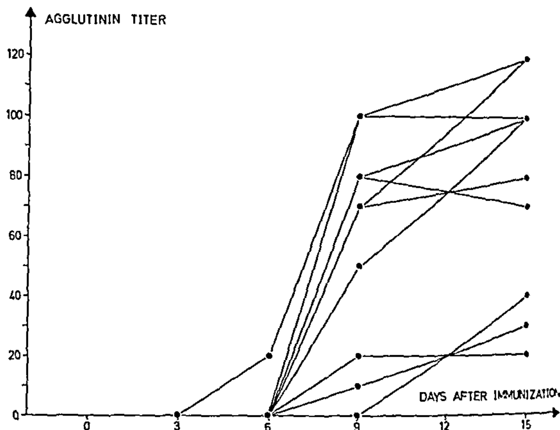


Fig 2 Serum agglutinin titres after immunization on day 0 in the 11 patients responding

of the other patients but escaping detection due to the limited number of samples

Four patients (Nos 3, 9, 14, 15) did not produce detectable amounts of circulating antibodies In most of the remaining cases, antibodies were detected on day 9 (Fig 2) The development of humoral hypersensitivity thus followed the well known pattern of rising titres after a latent period of approximately one week

It is apparent from these results that cellular and humoral hypersensitivity developed in two qualitatively different fashions Furthermore Table 1 shows that there is no quantitative correlation between the agglutinin titres and the migration indices observed in the individual patients

When the results of the leucocyte migration test and the serum agglutinin measurements are compared to the findings in the lymphocyte transformation studies presented in the preceding article (*Andersen et al* 1971) the following observations can be made

1) The development of lymphocyte transformation follows a pattern qualitatively different from both the leucocyte migration test and the agglutination test The early biphasic pattern of leucocyte migration inhibition (Fig 1) was not found in the transformation studies On the other hand transformation was apparent several days before agglutinins could be demonstrated in the serum

2) In the individual patients there was no quantitative correlation between lymphocyte transformation and migration inhibition As regards lymphocyte transformation versus agglutinin titres the poor responders tended to be identical in the two systems but otherwise no correlation was found

DISCUSSION

In the transformation experiments presented, autologous serum was present in the culture medium, in order to make the results comparable to those in the literature, most of which were obtained with autologous serum It must be emphasized that in the leucocyte migration test, autologous serum is not present in the medium This raises the possibility that in the transformation test, antibodies present in the serum may influence the lymphocyte response since it has been shown that antigen antibody complexes may induce lymphocyte transformation (*Hirschhorn et al* 1967, *Möller* 1969) However, in animals showing humoral hypersensitivity only, antigen induced transformation of lymphocytes has been demonstrated in cultures without autologous serum (*Loewi et al* 1968)

It has previously been shown (*Søborg* 1967, *Clausen* & *Søborg* 1969) that the size of the delayed intracutaneous reaction is reflected in the degree of inhibition of leucocyte migration, so that this *in vitro* test constitutes a quantitative as well as a qualitative correlate to delayed hypersensitivity From the present data it appears that, with the method of immunization employed, the lymphocyte transformation does not correlate with cellular hypersensitivity as expressed by the leucocyte migration test Only a poor correlation between transformation and humoral hypersensitivity was found, as expressed by the level of agglutinating antibodies in serum

A similar poor correlation between lymphocyte transformation and both cellular and humoral hypersensitivity has been demonstrated by *Benezra et al* 1969 in experiments with rabbits immunized with different antigens and according to different schedules The lymphocyte transformation response was compared to the magnitude of the Arthus reaction the delayed intracutaneous reaction and the antibody titre One general trend only was found namely that rabbits with strong immune responses usually showed more intense transformation reactions than those with weak immunity In the same experiments were included a group of rabbits which had circulating antibodies but showed neither positive Arthus reaction nor delayed intracutaneous reaction, nevertheless, these animals had marked lymphocyte transformation activity, proving that the lymphocyte transformation indeed may be positive without demonstrable delayed hypersensitivity

In a study of the response of normal individuals and cancer patients to immunization with keyhole limpet haemocyanin, *Curtis et al* 1970 found no correlation between lymphocyte transformation and delayed intracutaneous reactions, but a quantitative correlation was established between lymphocyte transformation and haemagglutinin titres, this was mainly due to the fact that the same poor responders showed up in each test Thus, our results are in agreement with these findings

On the basis of the data presented, the following hypothesis seems plausible The antigen induced lymphocyte transformation probably reflects the expanded clones of antigen sensitive cells raised after immunization whether the final immune response is antibody formation, cell mediated hypersensitivity or both

We wish to thank Mrs *Lis Jensen* for competent technical assistance

Supported by *The Danish Medical Research Council*, grant No 512 230/69, *Fonden til Lægevidenskabens Fremme*, and *Forskningsfonden for Storkøbenhavn, Færoerne og Grønland*

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Acta path microbiol scand Section B 79, 502–510 1971

THE HERPES SIMPLEX VIRUS ENCEPHALITIS IN MICE AT DIFFERENT ENVIRONMENTAL TEMPERATURES

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Mice inoculated intracerebrally with herpes simplex virus were kept at different environmental temperatures The life span, symptomatology, synthesis of infective virus, interferon and monoamines of the brains as well as the histopathological picture were studied Increased life-span and increased numbers of survivors were observed when mice were incubated at temperatures higher than the ordinary room-temperature At the higher temperatures the pathological rise in dopamine synthesis observed in mice at room temperature almost disappeared and the incidence of excited animals was markedly reduced The development of neuronal degenerations and pathological vascular permeability was inhibited in mice incubated at 34° in comparison to these conditions in mice kept at 22°, whereas no effect on the inflammatory cell response was demonstrable These effects of the higher environmental temperature were probably not mediated by increased production of interferon Although it is probable that the symptomatology modified by increased temperature was influenced by changes in metabolism of brain monoamines, the life-saving result of increasing the environmental temperature seemed dependent upon inhibition of formation of infective virus and of spread of the infection

Changes of the environmental temperature might result in a markedly modified resistance to infection In viral infections this can be observed with unicellular as well as multicellular hosts (7) Mice kept at 37°, instead of 24°, showed increased resistance to infection with herpes simplex virus (HSV) and survived to a high percentage 3, 19) In mice kept at 37° the rectal temperature was raised to 39 3°–40° and *Schmidt and Rasmussen* (1960) attributed the altered resistance to an impaired multiplication of virus in brains of mice maintained at the higher temperature An inverse relationship between virus and interferon production in mice has been reported inter alia by *Heineberg et al* (1964) and *Ruiz Gomez and Sosa-Martinez* (1965) In addition to the inverse relationship, *Ruiz Gomez and Sosa-Martinez* demonstrated that the environmental temperature influenced the production of interferon Abrupt changes of the environmental temperature will however also cause a stress condition in exposed animals Stress is reported to inhibit interferon production in virus infected mice (5) and increased body temperature to affect release of monoamines of the brain (6) *Jensen* (1968) has reported that administration of serotonin (5-HT) leads to a transitory impairment of interferon production in mice Recently, it was reported that intracerebral infection of mice with HSV caused raised release and synthesis of the brain monoamines dopamine, noradrenaline and serotonin (13 14

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The influence of the conditions mentioned on the course of infection was further studied in the present report The multiplication of HSV in the mouse brain and the synthesis of interferon and of brain monoamines were observed and the results were compared with observations of life span and histopathology of mice kept at different temperatures during the infection

MATERIAL AND METHODS

Virus The herpes simplex virus (HSV) strain used was originally isolated at the National Bacteriological Laboratory in Stockholm from a human case with oral herpetic lesions It was typed as a type 1 strain in our laboratory and used in the twelth mouse brain passage as a 20 per cent brain suspension with a titre of 4 8 × 10^7 plaque forming units (pfu) per ml Virus infectivity was titrated in monolayer cultures of green monkey kidney (GMK) cells The cells were cultivated in 5 cm plastic petri dishes with an overlay of Eagle s medium (MEM) supplemented with 3 per cent calf serum antibiotics and 1 per cent methyl cellulose Plaques were counted on the fourth day after the inoculation and titres expressed in pfu per ml

Mice Swiss albino mice 22 23 days old and of own laboratory breed were used The mice were killed by ether narcosis for assays of infective virus and monoamines and anaesthetized by ether and killed by decapitation for histological studies of the brains

The body temperature (rectal) was determined by means of a 0 5 mm thermistor connected with a calibrated instrument for optical registration with an accurary of 0 05°

Monoamines Determinations of dopamine (DA) and homovanillic acid (HVA) were made spectrophotofluorometrically according to *Carlsson and Lindquist* (1962) and *Andén et al* (1963) Assays of [illegible]

respectively The accuracy of the determinations has been reported previously (*Lycke et al* 1970)

Interferon The interferon of HSV infected mouse brains was assayed on groups of 5 brains ground in a mortar and suspended in 7 ml of MEM The disintegrated brain material was centrifuged at 2 000 r p m for 10 minutes and the supernatant dialysed overnight at 4° against 0 1 M HCl KCl buffer, pH 2 and against Hanks balanced salt solution to bring the pH to about 7 4 Precipitated material which appeared during acid dialysis was removed by centrifugation at 2 000 r p m for 10 minutes Twofold dilutions of the dialysed material in MEM were inoculated in amounts of 5 ml into groups of 2 mouse cell cultures, grown in plastic petri dishes The cultures were incubated at 37° for about 24 hours the medium removed and the cells challenged with approximately 100 pfu of vaccinia virus The virus was adsorbed at 37° for 1 hour and the cells overlayed with 5 ml MEM containing 1 per cent methylcellulose Plaques were counted after a further incubation of the cell cultures at 37° for 3 days The interferon titre was expressed as the reciprocal of the dilution that reduced the plaque number by 50 per cent

Histopathology The heads were fixed in Heidenhain's Susa overnight before the skulls were opened and the brains removed The brains were cut in horizontal planes through the cerebral hemispheres and in frontal planes through the pons The specimens were embedded in paraffin and sections, 10 μ thick, were cut These were stained with Giemsa and with luxol fast blue cresyl violet for myelin sheaths and Nissl substance

With a view to indicate the vascular permeability the method introduced by *Steinwall and Klatzo* (1966) was used Mice were injected intraperitoneally with 0 3 ml of a 1 per cent solution of Evans blue bound to 5 per cent bovine serum albumin 4 hours before they were sacrificed They were fixed by perfusion of saline through the left cardiac chamber for 1 2 minutes followed by bromformol The following day the skulls were opened and the brains removed Frozen horizontal sections, 10 μ thick were cut mounted in 50 per cent aqueous glycerol and immediately examined in a Zeiss fluorescence microscope equipped with a dark field condensor and an Osram HBO 200 W high pressure mercury lamp The light was filtered through a Schott BG 12/3 filter and in the tube the emitted light was filtered through a combination of a Schott OG4 and GG4 filter From the same brains sections were stained according to Cajal for astrocytes after the brains had been fixed in bromformol for a further 3 or 4 days

On each of the 5 days following inoculation, 6 brains of mice kept at 22° and 6 brains of those maintained at 34° were examined two brains being examined for vascular permeability and astrocytes the others for neuronal lesions and inflammatory cell response As controls served untreated mice and mice injected intracerebrally with 0 03 ml of saline Altogether 80 mice were examined

In order to evaluate the degree of neuronal lesions the following regions of both hemispheres were examined frontoparietal and occipitotemporal cortex, olfactory bulbs hippocampal region including both the pyramidal band and fascia dentata septal areas neostriatum, thalamic nuclei substantia nigra, cerebellum and pontine nuclei The neuronal lesions were graded according to a five

bolites of DA and 5 HT, but as the amines are rapidly metabolized to acids, the levels of the amines themselves are relatively unaffected.

The effect of changes in the environmental temperature on release and synthesis of monoamines was studied by assaying DA, 5 HT, NA, HVA and 5 HIAA concentrations in brains of mice kept at 4°, 22°, 34° or 37°, respectively. The mice were inoculated intracerebrally with 300 LD_{50} of HSV and the brains were harvested after varying periods of time. The concentrations of amines and acids observed were compared with those observed in uninfected animals (controls) incubated in parallel with the infected ones and with means calculated on the basis of series of tests with infected or uninfected brains (standards). A representative experiment is shown in Table 2.

Deviations of controls from standards all fell within the range of one σ. In infected mice kept at 4° or 22°, the brain contents of HVA were significantly higher than those encountered in uninfected animals indicative of a raised release of DA in the HSV infected animals at these temperatures. Such an increased release would normally stimulate a rise in synthesis of the amine and it is known from an earlier study (14) that this occurs in mice kept at 22°. From the low DA and NA concentrations observed in the infected mice incubated at 4° this seemed, however, not to have occurred at the low temperature.

If the temperature were raised above 22° the HSV infection seemed to cause no changes in DA synthesis. On the other hand the formation of 5 HT was increased in infected mice irrespective of the environmental temperature at which the animals were studied.

Virus Multiplication and Synthesis of Interferon

The effect of environmental temperature on multiplication of HSV in the mouse brain was studied by assaying the virus yields of brains harvested at varying times after the virus inoculation. Each time 5-10 brains were harvested, pooled and homogenized and the amounts of infective virus in 20 per cent brain suspensions were determined by titrations of pfu. Two environmental temperatures, 22° and 34°, were studied and all the mice were infected with 300 LD_{50} of virus.

Fig. 3 illustrates that maximal virus yields were observed in mice incubated at 22° 4-5 days after the inoculation, i.e. concomitant with the occurrence of deaths among the animals. As regards animals kept at 34° the virus growth curve demonstrated a slower initial rate and yields which were only about 10 per cent of those produced at 22°. Maximal yield was obtained after 5 days of infection. From then on successively reduced contents of infective virus were demonstrable in the brains.

The production of interferon in the brains is illustrated in Table 3. At both temperatures

TABLE 3. *Production of Interferon in B[illegible] of Mice after Intracerebral Infection with HSV and Incubation at Different Temperatures*

Day after inoculation	Interferon titre β	
	[illegible]°	37°
1	<10	<10
2	<10	<10
3	10	10
4	[illegible]	40
5	[illegible]	30
6		30
8		<10
10		<10

β) 5 homogenized mo[illegible]e brains suspended in [illegible] ml of MEM

TABLE 4. *Effect of In[illegible] on Plaque Formation of HSV in Mouse Embryo Cells Incubated at 37.0° C and 39.2° C*

Dilution of interferon	pfu per cent of control	
	[illegible]	39.2°
1/20	[illegible]	19
1/40	[illegible]	25
1/80	[illegible]	59
1/160	[illegible]	84

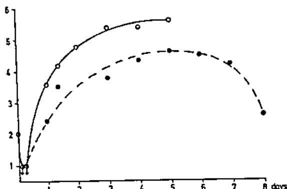


Fig 3 Virus multiplication in brains of mice intracerebrally infected with HSV and kept at 22° (solid line and open circles) or at 34° (dashed line and filled circles)

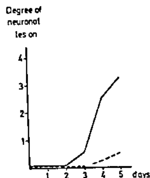


Fig 4 Neuronal lesions in brains of mice inoculated intracerebrally with HSV Solid line represents results obtained in mice maintained at 22°, dashed line results obtained in animals kept at 34° Scale of evaluation of degree of lesions is described in the text

studied, the production of interferon seemed not far from paralleling the virus growth curves, showing a maximum at about the same time as that when the largest virus yields were found The interferon titres were 3-4 times higher in brains from mice kept at 22° than in brains of animals kept at 34° Assays of sensitivity of HSV to interferon in mouse embryo cells indicated that the virus strain used might be slightly more sensitive at 39 2° than at 37° (Table 4)

Histology The morphological changes induced were studied in two groups of intracerebrally infected mice in one group the mice were kept at 22°, in the other at 34° Except for the area around the needle track, no signs of neuronal lesions were seen during the first two days after the virus inoculation On the third day, 2 of the 4 brains of mice kept at 22° showed small areas of neuronal degeneration confined to fascia dentata on one side These early neuronal lesions were characterized by dispersion of the Nissl bodies, decreased cytoplasmic basophilia and development of intranuclear eosinophilic inclusion bodies In brains harvested a day later, moderate to more extensive neuronal degeneration was seen and on the fifth day of infection there were pronounced and wide-spread lesions the neurons being necrotic or having disappeared

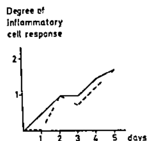


Fig 5 Inflammatory cell response in brains of mice inoculated intracerebrally with HSV Solid line represents results obtained in mice maintained at 22°, dashed line results obtained in animals kept at 34° Scale of evalution of degree of lesions is described in the text

The regions most commonly affected were the olfactory bulbs, the pyramidal band, the fascia dentata and the thalamic nuclei Lesions were seen only occasionally in the septal area, pontine nuclei, substantia nigra and cerebellum, while the cerebral cortex and neostriatum were generally spared

A slight infiltration of mononuclear inflammatory cells was seen in the leptomeninges one day after infection The following days the inflammatory cell response became more intense, resulting in a moderate infiltration into the meninges, choroid plexus and

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IMMUNITY TO *ASCARIS SUUM*

5 *The Effect of X-Radiation and Neonatal Thymectomy on a Primary Infection in Mice*

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The presence of immunological factors active in the regulation of the magnitude of a primary infection with *Ascaris suum* was investigated in X irradiated mice and in neonatally thymectomized mice More larvae developed in thymectomized mice than in their controls, but fewer larvae were recovered from irradiated mice than from the controls It is suggested that some thymus dependent innate immunity against *Ascaris suum* may be present in mice The radiation is supposed to have rendered the mice unsuitable hosts for the parasites

Highly energetic rays, e g X and γ rays, may interfere with the immunological response and are therefore often applied in studies of the antibody response to different kinds of antigens (see *Taliaferro et al* 1964)

The use of radiation may, however involve a number of disadvantages It is for example important to realize that radiation will interfere with both the cellular response and the development of circulating antibodies thus making it very difficult to distinguish between the two functions Additionally many different kinds of cells in the organism and not only the immunologically competent cells and their precursors are affected by the radiation The use of radiation also causes a more practical difficulty as a relatively high dosage of radiation may be necessary to get a significant effect on the immune response, whereby the survival times of the animals may be so short that the animals may die before the experiments can be properly terminated

In spite of these disadvantages radiation treatment is widely used in studies of infectious diseases Concerning its application to experimental parasitology the reader is referred to reviews by *Casarosa* (1967, 1968), in which several works on helminth infections are reviewed

In parasitic infections, the effect of radiation prior to infection is generally marked by an increased lethality following the infection (*Stoner & Hale* 1952, *Yarinsky* 1962), and development of a greater number of parasites (*Dunsmore* 1961) On the whole, radiation will make animals more susceptible to infection (see *Taliaferro et al* 1964)

The influence of radiation on immunity to *Ascaris suum* has been studied particularly by Italian workers, viz *Casarosa* (1968 1969, 1970), *Casarosa & Macchioni* (1969) and *Casarosa et al* (1967) who used guinea pigs They concluded from their experiments that X radiation at LD 50/30 — almost equal to 400 r — caused a higher death rate *in irra-*

Received 19 xii.70

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diated, infected animals than in animals only irradiated or only infected. However, radiation did not increase susceptibility to a challenge infection but acquired immunity was abolished by radiation.

By neonatal thymectomy an influence almost exclusively on cellular immunity is achieved thus making it a useful tool by which to distinguish between cellular immunity due to circulating antibodies. By using neonatally thymectomized mice it has been shown that thymus dependent factors are involved in the immunity to *Hymenolepis nana* (*Okamoto* 1970).

Results of previous investigations have suggested that the initial development of *Ascaris suum* in conventional mice may be regulated by some immunological factors (*Bindseil* 1970 a). The present work was undertaken to investigate the importance of immunological factors on the initial migration of *Ascaris suum* in mice by studying the course of infections in X irradiated mice (Experiment 1) and thymectomized mice (Experiment 2).

EXPERIMENT 1

MATERIALS AND METHODS

Mice. Male conventional mice and male SPF mice (both N.M.R.I.) weighing 16–18 g, purchased at 2 different breeding centres, were used.

Ascaris suum eggs for infection. The preparation of the *Ascaris* eggs was carried out as described earlier (*Bindseil* 1969 a). The eggs were administered with the aid of a stomach tube while the mice were slightly anaesthetized with ether.

X radiation. The mice in question were exposed to whole body radiation from *Siemens* Stabiliplan at 46 r/min with a total dose of 600 r with a 0.5 Cu filter and at 16 mA and 200 kV. The radiation was carried out at the *Radio physical Lab.*, the *Radium Centre*, the *Finsen Hospital*, Copenhagen.

Post mortem examination. At necropsy all mice were treated in the same way. The mediastinal lobes of the lungs, the right lobes of the livers and the caecum were removed and fixed in 10 per cent formalin and embedded in paraffin. Then they were treated and stained as described elsewhere (*Bindseil* 1969 b). Sections of the caecum were furthermore stained with toluidine blue to detect mast cells.

EXPERIMENTS

The conventional mice as well as the SPF mice were divided into 2 groups. Thus 4 groups of mice were included in the experiments out of which one group (SPFC) consisted of 20 SPF mice serving as non irradiated controls. Another group (SPFX) consisted of 20 SPF mice which were irradiated. Corresponding groups of conventional mice were included and called CC and CX respectively. All mice were challenged with 3 000 eggs 4 days after the radiation. On each day for 4 days after challenge 3 mice from each group were sacrificed and examined.

RESULTS

Clinical Observations

During the first 3–4 days following the X radiation no clinical sign of a radiation syndrome was observed. From that time a decreased food intake and an increasing lethargy were observed in the irradiated mice. No diarrhoea was seen. From the 7th day following the radiation (the 3rd day following challenge) the irradiated mice had a high death rate compared to the non irradiated mice, where only one mouse died spontaneously within 14 days.

Post Mortem Examination

Gross lesions. During the experimental period, the only difference between irradiated and non irradiated mice was that large confluent haemorrhages were seen on the lungs of the former. Moreover small bowels containing bile stained contents were conspicuous in irradiated animals. The livers of all mice were greyish brown with minute grey spots on the surface.

Recovery of larvae. The course of the challenge infection in the liver during the experimental period is outlined in Fig. 1 showing the average number of larvae recovered each day. In accordance with previous experiments (*Bindseil* 1969 b, 1970 a, b) the numbers of lung larvae were very low, less than 100 larvae on average per mouse, and consequently no further description or discussion of these figures will be given.

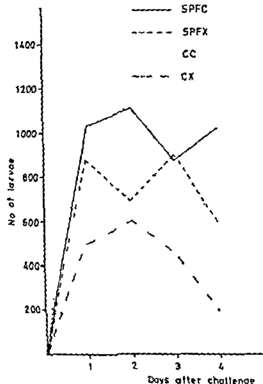


Fig 1 Distribution of *Ascaris suum* larvae in the liver ($^1/_1$ organ) of mice during the first 4 days of an infection with 3 000 *Ascaris suum* eggs (average of 3 mice) Irradiated SPF mice (SPFX) irradiated conventional mice (CX) and non irradiated controls — SPFC and CC respectively

It appears from Fig 1 that the rate of migration of larvae in the irradiated conventional mice was much lower than that in the other groups especially than in the 2 non irradiated groups No significant difference between the 2 control groups seems to exist Despite the differences in numbers of larvae recovered the migratory pattern of the larvae does not seem to have been altered by the X radiation

HISTOLOGY

a) *Caecum*

The caecum of all mice largely showed the same histological features Generally the cellular infiltration was slight The infiltration consisted of eosinophils pyroninophils lymphocytes macrophages and a few mast cells No larvae could be demonstrated

b) *Liver*

1) *Non irradiated mice* No details of the histological picture will be given as they are in principle similar to those described earlier (*Bindseil* 1969 b) The damage was as previously found increasing in severity from the 1st day following challenge till the 4th day, which was the last day of the experiment Larvae were normally not surrounded by any inflammatory reaction No significant differences between conventional mice and SPF-mice were noticed

2) *Irradiated mice* No essential difference between the 2 irradiated groups was noticed The liver damage was still increasing from day one to day four, but there was a very conspicuous difference between reactions in

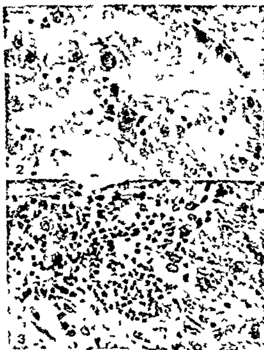


Fig 2 Section of liver from mouse 4 days after challenge with 3 000 *Ascaris suum* eggs, and 8 days after X radiation at 600 r Most parenchyma necrotic due to larval migration Note almost complete absence of cellular reaction HE 400 ×

Fig 3 Section of liver from non irradiated mouse 4 days after challenge with 3 000 *Ascaris suum* eggs Marked infiltration with cells around and within necrotic parenchyma HE 400 ×

the liver of these mice and in that of the non irradiated controls (Figs 2 and 3) Histologically there was almost no apparent host reaction to the damaged liver tissue in irradiated mice throughout the experimental period The areas of haemorrhage and necrosis were lying almost without any sign of cellular reactions in the surroundings

c) Lung

The histological picture of the lungs was practically similar in all 4 groups The changes were slight and consisted mainly of perivascular and peribronchial accumulations of lymphatic tissue, histiocytes and some plasma cells and eosinophils Thus the features were like those found in normal conventional mice

The observations that the number of larvae to develop in non irradiated conventional mice was practically the same as that to develop in non irradiated SPF mice and that there was no apparent delay in the occurrence of larvae in the former group were not in accordance with previous results (*Bindseil* 1969 b 1970 a) The latter study had shown infections of greater magnitude in SPF mice than in conventional ones and was *inter alia* carried out because a previous preliminary experiment including 8 SPF mice and 8 conventional controls had shown that the number of larvae to develop in the SPF group was about three times as high as that in the group of conventional mice

In order to test a primary infection with *Ascaris suum* in SPF mice and conventional mice and to test the rather unexpected lowered susceptibility in X irradiated mice a third experiment (Experiment 1 a) identical with Experiment 1 was carried out

In Experiment 1 a the irradiated conventional mice showed a very high death rate as no mice survived the 7th day following the radiation whereas none of the irradiated SPF mice died The latter however showed an increasing degree of lethargy The *post mortem* findings and the histological pictures were in principle identical with those in Experiment 1 It appears from Fig 6 that there is some tendency towards higher larval counts in the SPF controls than in conventional controls but the difference is not very marked but still no delay in the occurrence of larvae in conventional mice is seen Again the irradiated conventional mice showed a decreased susceptibility to the infection and this is valid too for the irradiated SPF mice but to a less extent

EXPERIMENT 2

MATERIALS AND METHODS

Mice Mice of a thoroughly inbred strain (S a) thymectomized neonatally were delivered from the *Fibiger Lab* Copenhagen at the age of 3–4 weeks Non thymectomized mice served as controls and both male and female mice were used The thymus was removed by sucking through a small incision in the sternum

Ascaris suum eggs for infection Eggs for infection were taken from the batch of eggs used in Experiment 1

Post mortem examination At necropsy all mice were treated as in Experiment 1

EXPERIMENTS

Twenty neonatally thymectomized mice and as many non thymectomized controls were challenged on the same day with 1 500 *Ascaris* eggs On each day for 10 days after challenge 2 mice from each group were sacrificed and examined

RESULTS

Clinical Observations

During the first days of the infection all mice behaved like normal uninfected mice but from the 5th day and onwards an increasing degree of dyspnoea and until [illegible] was observed in both groups obviously ascribable to the pulmonary migration of the *Ascaris suum* larva

Post Mortem Examination

Gross lesions The livers were mottled with minute grey spots The lungs appeared normal till the 4th 5th day after challenge and from that time an increasing number of pe

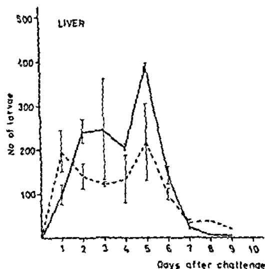


Fig 4 Distribution *of Ascaris suum* larvae in the liver ($^1/_3$ organ) of thymectomized mice (———) and non thymectomized *controls* (– – – –) after challenge with 1 500 *Ascaris suum* eggs (average of 2 mice) Variances indicated by vertical bars

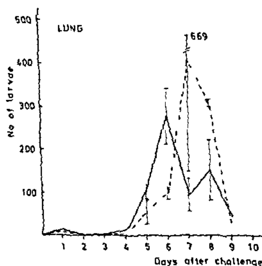


Fig 5 Distribution of *Ascaris suum* larvae in the lung ($^1/_3$ organ) of the same mice as in *Fig 4* Variances indicated by vertical bars

techiae was noticed on the surface In no case any remnants of thymus were seen macroscopically in the thymectomized group

Recovery of larvae In Figs 4 and 5 the average numbers of larvae in liver and lung are seen It appears from the liver figures (Fig 4) that more larvae have developed in thymectomized mice than in the controls, and that is reflected also in the lungs during the first 6 days of the infection (Fig 5) Later more larvae are recovered from the controls Nothing indicated that the differences between the 2 groups observed during the infection might be due to the fact that both male and female mice were used in the experiment

Histology The histological manifestations in the caecum, liver and lung were generally very similar in the 2 groups, thus the thymectomy apparently did not affect these organs histologically

The changes will not be described in detail as they are quite similar to those described in the preceding experiment (non-irradiated mice) Larvae in the livers were not surrounded by cellular reactions

DISCUSSION

It was decided to use a relatively high dosage of X-radiation to get a conclusive effect on the immune response, in which case it was evident, however, that survival of the mice would hardly be sufficiently long to allow a study of the pulmonary migration of the *Ascaris* larvae Consequently the attention was focused on the initial 4 days of the infection as the magnitude of the infection is determined within that time (*Bindseil* 1969 b, 1970 a, b,) It appears from the high death rate among irradiated animals, and from the almost complete absence of a cellular reaction in the liver of these mice, that the radiation has had a marked effect on the organism of the mice The finding of an increased death rate in irradiated animals is in accordance with the observations on guinea pigs by *Casarosa et al* (1967) and *Casarosa* (1968)

The migratory patterns (Figs 1 and 6) were the same as described earlier (*Bindseil* 1969 b, 1970 a, b) indicating that these patterns have not been changed by the radiation Consequently, the differences in the numbers of larvae recovered show that the

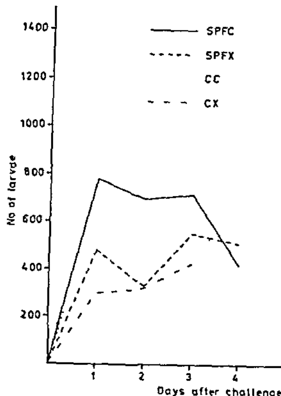


Fig 6 Repetition of Experiment 1 (Fig 1) Distribution of *Ascaris suum* larvae in the liver (¹/₁ organ) of mice during the first 4 days of an infection with 3 000 *Ascaris suum* eggs Same legend as in Fig 1

radiation has caused an increased resistance to the *Ascaris* infection The difference was more pronounced in the conventional mice than in the SPF mice The reason why the differences between the 2 non irradiated groups were not as marked as expected is not clear It was obvious to suggest that the SPF status had been broken down as the mice spent up to 7 days at the author s laboratory but the theory is not reasonable as the SPF mice in the preliminary experiment had spent more than 8 days at the laboratory

Earlier *Casarosa et al* (1967) and *Casarosa* (1968) found that X radiation did not increase susceptibility to *Ascaris suum* in guinea pigs It seems evident considering these and the preceding results that the effect of radiation is dependent on the dosage of radiation on the species of the animals and probably also on the species of the parasites

In that respect the harmful effect of radiation on the body should be remembered

It is evident that the physiology of the intestine is changed during the gastro-intestinal syndrome *Rogers* (1962) claims that the establishment of an infection with intestinal parasites is dependent on certain physicochemical features in the intestine One can say that the host acts as a culture medium for the parasites If the medium is significantly altered it may hinder the establishment of the infection

As regards the present study with *Ascaris suum* it is pertinent to remember that especially eggs situated near the mucosa will hatch (*Ibragimov* 1957, according to *Fu ey* 1963), and that especially larvae near the mucosa will start migration (*Bhowmick* 1964) The normal course of these events is obviously dependent on certain well-defined features in the habitat, i e the surface of the mucosa which is particularly radiosensitive Undoubtedly the intestine of the irradiated mice has been physiologically altered and not only the small intestine as judged from the gross lesions but probably also the caecum although histo-pathological changes were not detected The absence of histological changes does not necessarily mean that the function is normal

The eggs administered to irradiated animals have reached a habitat which has been unfavourable to the hatching and the larval penetration i e the heavily irradiated mice are unsuitable hosts for *Ascaris suum* just as heavily irradiated rats are found to be unsuitable hosts for *Nippostrongylus brasiliensis* (*Ogilvie* 1970 *Taliaferro et al* (1964) stressed that disturbances in the host parasite relationship have to be considered when the effects of radiation on infections are to be estimated

No observation indicated that the thymectomized mice were different from the controls except for the lack of thymus In addition nothing indicated that the use of both males and females had interfered with the results Consequently it is suggested that some degree of thymus dependent innate immunity

to *Ascaris suum* is present in mice These results do not exclude the possibility of circulating antibodies (IgM, IgG) being active also Thus unspecific immunological factors seem not only to be capable of inducing an acquired immunity to *Ascaris suum* in mice as reported earlier (*Bindseil* 1970 c), but immunological factors seem also to be capable of interfering with the establishment of a primary infection

The results show furthermore that the innate immunity is working in relation to the intestine as the acquired immunity does (*Bindseil* 1969 b, 1970 b) In the latter work it is stated that the acquired immunity is working in the intestinal lumen Thymectomy is known to reduce the development of anaphylactic antibodies in rats (*Wilson et al* 1967) and that may hint that these antibodies may be underlying the mechanism of innate immunity to *Ascaris suum* in mice The possible importance of anaphylactic antibodies (reagin like antibodies) for immunity has been suggested in *Nippostrongylus brasiliensis* infections in rats by *Ogilvie* (*1967*) but recently it has been demonstrated that reaginic antibodies are not essential for the immunity (*Jones et al* 1970) At first sight the involvement of cellular immunity to the parasitic stages in the lumen of the gut does not seem probable but that appears worth while studying as *Hopkins* (1970) has provided good evidence that cellular immunity is involved in the immunity to the tapeworm *Hymenolepis diminuta* in mice Circulating antibodies may also be involved as immunity to *Hymenolepis nana* in mice can be transferred passively with serum (*Hearin* 1941)

The hypothesis has been put forward that factors responsible for the acquired immunity to *Ascaris suum* and for the delay in the initial development of larvae in non immune conventional mice are similar in nature (*Bindseil* 1970 a) The present results seem not to give further knowledge of that and they have not shown that these factors are necessarily identical with the factors restricting the larval migration in conventional mice

Admittedly, extensive studies of the intestinal mucosa and its surface as a habitat for *Ascaris* eggs and larvae under different conditions are required before one can get any further idea of the mechanisms which are released in the mucosa and which are, undoubtedly, determining the magnitude of the infection in immune and non immune hosts

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The author is greatly indebted to Mr *J Ambrosen* the *Radio-physical Lab* the *Radium Centre* the *Finsen Hospital* Copenhagen where the X radiation was carried out and to Mr *J Kieler* M D the *Fibiger Lab* Copenhagen who supplied the thymectomized mice and their controls

The work was carried out with financial support from the *Danish Agricultural and Veterinary Research Council*

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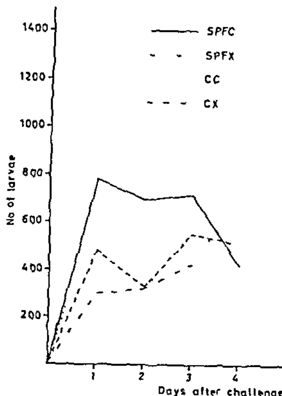


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The results show furthermore that the innate immunity is working in relation to the intestine as the acquired immunity does (*Bindseil* 1969 b, 1970 b) In the latter work it is stated that the acquired immunity is working in the intestinal lumen Thymectomy is known to reduce the development of anaphylactic antibodies in rats (*Wilson et al* 1967) and that may hint that these antibodies may be underlying the mechanism of innate immunity to *Ascaris suum* in mice The possible importance of anaphylactic antibodies (reagin like antibodies) for immunity has been suggested in *Nippostrongylus brasiliensis* infections in rats by *Ogilvie* (1967) but recently it has been demonstrated that reaginic antibodies are not essential for the immunity (*Jones et al* 1970) At first sight the involvement of cellular immunity to the parasitic stages in the lumen of the gut does not seem probable but that appears worth while studying as *Hopkins* (1970) has provided good evidence that cellular immunity is involved in the immunity to the tape worm *Hymenolepis diminuta* in mice Circulating antibodies may also be involved as immunity to *Hymenolepis nana* in mice can be transferred passively with serum (*Hearin* 1941)

The hypothesis has been put forward that factors responsible for the acquired immunity to *Ascaris suum* and for the delay in the initial development of larvae in non immune conventional mice are similar in nature (*Bindseil* 1970 a) The present results seem not to give further knowledge of that and they have not shown that these factors are necessarily identical with the factors restricting the larval migration in conventional mice Admittedly, extensive studies of the intestinal mucosa and its surface as a habitat for *Ascaris* eggs and larvae under different conditions are required, before one can get any further idea of the mechanisms which are released in the mucosa and which are, undoubtedly, determining the magnitude of the infection in immune and non immune hosts

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The author is greatly indebted to Mr *J Ambrosen* the *Radio physical Lab* the *Radium Centre*, the *Finsen Hospital*, Copenhagen where the X radiation was carried out, and to Mr *J Kieler*, M D the *Fibiger Lab* Copenhagen, who supplied the thymectomized mice and their controls

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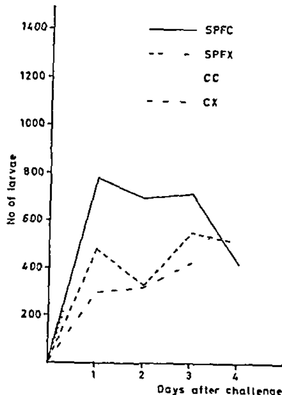


Fig 6 Repetit on of Experiment 1 (Fig 1) Distribution of *Ascaris suum* larvae in the liver (1/1 organ) of m ce durin g the first 4 days of an infection with 3 000 *Ascaris suum* eggs Same legend as in Fig 1

radiation has caused an increased resistance to the *Ascaris* infection The difference was more pronounced in the conventional mice than in the SPF mice The reason why the differences between the 2 non irradiated groups were not as marked as expected is not clear It was obvious to suggest that the SPF status had been broken down as the mice spent up to 7 days at the author s laboratory but the theory is not reasonable as the SPF mice in the preliminary experiment had spent more than 8 days at the laboratory

Earlier *Casarosa et al* (1967) and *Casarosa* (1968) found that X radiation did not increase susceptibility to *Ascaris suum* in guinea pigs It seems evident considering these and the preceding results that the effect of radiation is dependent on the dosage of radiation on the species of the animals and probably also on the species of the parasites

In that respect the harmful effect of radiation on the body should be remembered

It is evident that the physiology of the intestine is changed during the gastro-intestinal syndrome *Rogers* (1962) claims that the establishment of an infection with internal parasites is dependent on certain physico-chemical features in the intestine One can say that the host acts as a culture medium for the parasites If the medium is significantly altered it may hinder the establishment of the infection

As regards the present study with *Ascaris suum* it is pertinent to remember that especially eggs situated near the mucosa will hatch (*Ibragimov* 1957 according to *Fu rby* 1963), and that especially larvae near the mucosa will start migration (*Bhow m k* 1964) The normal course of these events is obviously dependent on certain well-defined features in the habitat, i e the surface of the mucosa which is particularly radiosensitive Undoubtedly the intestine of the irradiated mice has been physiologically altered and not only the small intestine as judged from the gross lesions but probably also the caecum although histo-pathological changes were not detected The absence of histological changes does not necessarily mean that the function is normal

The eggs administered to irradiated animals have reached a habitat which has been unfavourable to the hatching and the larval penetration i e the heavily irradiated mice are unsuitable hosts for *Ascaris suum* just as heavily irradiated rats are found to be unsuitable hosts for *Nippostrongylus brasiliensis* (*Ogilvie* 1970 *Taliaferro et al* (19) stressed that disturbances in the host parasite relationship have to be considered when the effects of radiation on infections are to be estimated

No observation indicated that the thymectomized mice were different from the controls except for the lack of thymus In addition nothing indicated that the use of both males and females had interfered with the results Consequently it is suggested that some degree of thymus dependent innate immunity

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after 7 days Some of the animals were then inoculated with new strains of meningococci whereas others were taken out of the isolators, killed, and samples for bacteriological examinations were taken from the oral cavity, heart blood, lungs, brain and peritoneal cavity Alltogether, 3 rats and 12 mice of which 4 animals were germfree, were investigated *post mortem* In none of the animals, however, *N meningitidis* could be demonstrated

DISCUSSION AND CONCLUSION

Previously, *Pollard et al* (6) have failed to establish strains of *N meningitidis* in germfree guinea pigs, rats and mice *Gibbons et al* (1), working with other microorganisms indigenous to man, demonstrated that difficulties in establishment of such microorganisms in germfree mice could be due to inhibitory substances in the diet The present results confirm those of *Pollard et al* (6) The dietary composition used seems to exclude the possibility of inhibitory substances in the diet It seems reasonable to assume that difficulties in establishing *N meningitidis* in gnotobiotic animals are due to endogenous factors

The authors are indepted to *Norsk Medisinaldepot* for financial support

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INTESTINAL GLYCOPROTEINS OF GERMFREE RATS

Chemical Composition of Intestinal and Fecal Mucus from Germfree Rats Fed a Chemically Defined Diet

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Intestinal and fecal contents were collected from germfree rats fed a chemically defined diet The contents considered to consist mainly of the epithelial mucin of the gastro intestinal tract, were separately extracted with water followed by dialysis and freezedrying The products obtained had a similar composition, corresponding to substances of glyco protein nature The carbohydrate part, constituting about 80 per cent of the material, contained galactose, mannose, fucose, arabinose, xylose, N acetylglucosamine, N acetyl galactosamine and sialic acid In the peptide moiety serine, threonine, aspartic acid and glutamic acid were the major amino acids Gel chromatographic experiments indicated chemical as well as physical heterogeneity of the extracts

The slimy substances found in the digestive tract of mammals will be partly exogenous, originating from the diet, partly derived from the microorganisms present, and partly endogenous, comprising the epithelial mucus which is secreted continuously by the intestinal lining The latter material more adequately might be termed a mucin indicating a mucus consisting mainly of glycoproteins Throughout the mammalian digestive tract there are different types of mucin-producing cells located to various regions of the gut wall (8) This mucin is believed to act as a lubricant for intraluminar mass movements, as a protective for the epithelial cells and it may also interfere with the absorption from the intestinal lumen into the mucosa Although abundant evidence exists that the secretions are of glycoprotein nature, not much appears to be known of whether the secretory products from different parts of the gut are of similar or of distinctly different composition Furthermore it seems far from clear to which extent the mucin undergoes chemical alterations by passing through the gut Such changes may be caused by endogenous factors, or more probably, by intestinal microorganisms

A characteristic feature of germfree animals is the presence of substantial amounts of mucoid material in the gastrointestinal tract (11, 12, 14) This accumulation is regarded as being due to the absence of the intestinal flora, normally degrading the macro molecular mucus to smaller fragments Most of the mucoid material present in germ free animals is of endogenous origin, but part of it might be derived from the diet By feeding germfree animals a defined diet

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DEVELOPMENT OF SULPHONAMIDE RESISTANCE IN *TOXOPLASMA GONDII*

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The RH strain of *Toxoplasma gondii* was transferred intraperitoneally in parallel in two different groups of mice One group received conventional food, the other the same diet plus sulphamethoxazole 0 5 mg per mouse daily The strain transferred on the mice fed sulphamethoxazole was designed the SR strain After transfers for 290 days the effect of sulphamethoxazole on infections caused by the SR strain was significantly less than on the infections caused by the RH strain It is concluded that a relative resistance to sulphamethoxazole may develop in *T gondii* when the protozoan is exposed to sublethal doses of the drug for long periods

In 1941 *Sabin* & *Warren* (2) found that sulphonamides were effective in the treatment of experimental toxoplasmosis in mice *Summers* (4) showed in 1947 that this effect could be reversed by concurrent administration of paraaminobenzoic acid or folic acid These results give support to the idea that sulphonamides interfere with folic acid biosynthesis in the protozoan They indicate that *Toxoplasma gondii* may synthesize its own folic acid but that it also can utilize preformed folic acid from the surroundings

When exposed to sulphonamides, originally sensitive bacteria can develop resistance both in vitro and in vivo So far, however, no reports have been presented concerning the development of such resistance in protozoan We hereby give a preliminary report of experiments which indicate that development of sulphonamide resistance may take place in *Toxoplasma gondii*

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MATERIALS AND METHODS

Toxoplasma Strain

The RH strain of *T gondii* was used in the experiments The sulphonamide resistant strain (SR strain) originated from the RH strain by transferring it on sulphamethoxazole fed mice

Animal Procedures

The strains were transferred on white mice of both sexes, wheighing about 20 grams each, being 60–80 days old Two parallel routine transfers of *T gondii* were performed The mice in both groups received the same standardized meals The mice, on which the SR strain was transferred, received in addition 0 5 mg sulphamethoxazole daily per mouse in their diet The peritoneal exudate was taken simultaneously in both groups on the fifth or sixth day after inoculation The exudate was diluted about 20 times, and aliquots of 0 5 ml were injected into the next series of mice The exudates were checked for purity by microscopical examinations and cultivations on blood agar plates

When testing for sulphonamide resistance only female mice were used The peritoneal exudate was taken on the fourth day in both groups Penicillin (1000 I U per ml) and streptomycin (100 mcg per ml) were added The exudates were then

TABLE 1 *Effect of Sulphamethoxazole on Experimental Toxoplasmosis in Mice Infected with Two Different Strains of T. Gondii*

Group	Cage no	No of mice per cage	No of mice total	Infected with strain	Daily treatment for 20 days	Day of death after inoculation	Survivors per cage after 20 days	No of [illegible] days per cage
A	1–6	10	60	SR	Sulphamethoxazole 1 mg	6,6,6,6,6,6,6,6,6,6,6,6,6,7,7, 7,7,7,7,7,7,7,7,7,7,7,7,7,7,7, 7,7,7,7,7,7,7,8,8,8,8,8,8 8 9,9, 9,9,10,10,11,11,17,18,18,20	1/10 1/10, 1/10, 1/10 1/10, 0/10	[illegible]
B	7–9	10	30	SR	None	5,5,5,5,5,6,6,6,6,6 6,6,7,7,7, 7,7,7,7,7,7,7,7,7,7,7,7,7,8,9	0/10, 0/10 0/10	62 72 62
C	10–15	10	60	RH	Sulphamethoxazole 1 mg	5 6,6,6,6,6,6,7,7,7,8,8,8 8,8, 8,9,9,9,9,10,10,10,11,13,13, 13,20,20	5/10, 6/10 4/10 5/10, 4/10, 7/10	139 1[illegible] 131 13[illegible] 142 1[illegible]
D	16–18	10	30	RH	None	1,5,5,5,5,6 6,6,6,6,6,6,6 6,6, 6,6,6,6,7,7,7,7,7,7,7,7,7,7,7	0/10 0/10, 0/10	65 [illegible] 64
E	19	10	10	None	None	9,9	8 10	178
F	20	10	10	None	Sulphamethoxazole 1 mg	6,6,8,9,13	5/10	142

Survival days per cage in group A less than in group C ($p < 0.005$), and in group B less than in group A ($p < 0.025$)

diluted to give 7 10^3 protozoans per ml Aliquots of 0 5 ml of this solution were then injected intraperitoneally into the test mice The chemotherapeutic treatment was initiated when the inoculation took place When testing for carrier state, a technique described elsewhere was used (3)

Chemotherapeutics

Sulphamethoxazole 5 methyl-3-sulphanilamido isoxazole was kindly supplied by Bourroughs Wellcome & Co London G B The drug was mixed daily in the food Sulphamethoxazole 0 5 mg daily given to the mice transferring the SR strain could not cure the mice, but should be sufficient to select sulphonamide resistant protozoans.

One mg daily per mouse was given in the final experiment Earlier experiments (3) have shown that about 50 per cent of the mice infected with the RH strain will survive 20 days on this treatment thus ensuring maximum statistic efficiency to the experiment

Statistics

The mice were randomized in each experiment The numbers of survival days per cage were used as statistic for the two-sample rank test

RESULTS

The RH and SR strains were transferred separately for 290 days Then an experiment was designed to compare the effect of sulphamethoxazole on the strains The results are presented in Table 1 It may be seen that sulphamethoxazole gives a better protection against infections with the RH strain than against infections caused by the SR strain The difference is highly significant ($p < 0.005$)

All the mice who survived in the sulphonamide treated group infected with the SR strain were carriers Among the survivors in the sulphonamide treated mice, infected with the RH strain 6 mice were randomly selected and tested for carrier state Three of these were carriers This difference is not significant ($p > 0.20$)

A comparison between the two groups of mice infected with the SR strain, shows that the sulphonamide treated group have a longer survival time than the untreated group This difference is significant ($p < 0.025$)

Untreated mice have about the same survival time whether they were infected with the SR strain or with the RH strain

DISCUSSION

The results demonstrate that the effect of sulphamethoxazole on infections with *T gondii* is significantly lessened if the infectious strain is exposed to sulphamethoxazole beforehand The mice who died were all seriously ill from toxoplasmosis and there were no differences in the survival time in the two untreated groups This makes it reasonable to assume that resistance against sulphamethoxazole developed in the protozoans exposed to sublethal doses of the drug for long periods

Therapeutic doses of sulphamethoxazole do however have an effect on infections with the SR strain The SR strain thus seems to be only partially resistant to sulphamethoxazole even after 290 days exposure to the drug

The mortality in the control group receiving sulphamethoxazole is probably caused by the high amounts of drug given However the difference between the groups of infected mice is not influenced by this mortality

The development of sulphonamide resistance in bacteria may occur by several mechanisms even in the same species (1) Further studies are in progress to clarify the details in the development of sulphonamide resistance in *T gondii*

The currently used laboratory strains of *T gondii*, sensitive to sulphonamides, were generally isolated before sulphonamides came into extensive use in clinical medicine It seems reasonable to assume that development of sulphonamide resistance may take place also in vivo In clarifying whether such resistance has any therapeutical significance in human toxoplasmosis it might be of importance to investigate the effect of sulphonamides on newly isolated strains of *T gondii*

This study was supported by a grant from *Norsk Medisinaldepot* Mr *P Torgersen* is thanked for skilful technical assistance

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Acta path microbiol scand Section B 79, 534–538, 1971

STUDIES ON ANTIBODY-CONTAINING CELLS IN LYMPH NODES AND THE LYMPH OF RABBITS IMMUNIZED WITH HUMAN IMMUNOGLOBULIN G ACCORDING TO VARIOUS SCHEDULES

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The results of the present study agree with the view that antibody synthesizing lymphoid cells spreading along the lymph route take part in the dissemination of the immune response At an immune response with a more localized antigenic stimulation of the lymphoid tissue, antibody-containing cells were demonstrable in cell populations from the efferent lymph of the regional lymph node, but never in the thoracic duct lymph However such cells were found in the thoracic duct lymph at an immune response with a more generalized antigenic stimulation of the lymphoid tissue contributing to the thoracic duct cell population The difference in content of antibody-containing cells in the thoracic duct lymph at the studied immune responses is discussed A *de novo* antibody synthesis of antibodies *in vitro* was conclusively proved in cultures of cell populations from the efferent lymph vessel and the thoracic duct

The dissemination of the immune response after different immunization methods has been extensively discussed in the literature One possibility is that immune responses induced by the injection of a soluble protein antigen, in the present study human IgG disseminate within the body by rapid spreading of the antigen Another possibility is that the dissemination takes place by means of sensitized lymphoid cells which carry the information of antibody synthesis to other lymphoid organs Thus the local injection of antigen could result in a spreading of sensitized lymphoid cells along the lymph route towards the thoracic duct

In agreement with the latter view, many previous studies recently reviewed by [illegible] (1969), have given more or less strong support for an antibody synthesizing capacity of lymphoid cells obtained from the efferent lymph vessel of the regional lymph node However none of these studies proved conclusively a *de novo* synthesis of antibodies in cultures of such cells

The opinions about the antibody synthesizing capacity of thoracic duct cell populations are controversial (for references see [illegible] 1969) In contrast to other authors *[illegible]*

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& *Sorkin* (1965), *studying the incorporation* of radioactively labelled amino acids into antibodies by co precipitation technique, de monstrated a *de novo* synthesis of antibodies in cultures of thoracic duct cell populations, but in only 2 out of 11 experiments As stressed by *Stavitsky* (1961) this sensitive method may contain significant sources of error due to non specific adsorption of radioactivity

The present investigation studied the spreading of antibody-containing and antibody-synthesizing lymphoid cells from lymph nodes along the lymph route towards the thoracic duct at an immune response with a more localized and one with a more generalized antigenic stimulation of the lymphoid tissue contributing to the thoracic duct cell population

MATERIAL AND METHODS

Antigen injections The toe pads of the hind paws of 55 rabbits were injected with a water in oil emulsion containing equal volumes of human IgG (AB Kabi, Stockholm) in aqueous solution and Freund s complete adjuvant (Difco, Detroit) Three of the rabbits were also injected intra venously with the antigen in aqueous solution

Experimental groups Three groups of rabbits were stud ed In the toe pads of the first group (5 rabbits) 6 μg of the ant gen was injected at day 0 and day 10 These were studied at day 14 The second group (47 rabbits) was treated in the same way, except that 6 mg of antigen was given at each injection In the third group (3 rabbits) 6 mg was injected into the toe pads Seven months later, a 10 mg intravenous antigen injection was given on three consecutive days Six months later, 10 mg antigen was again injected intravenously Four days after the latter injection e g about 13 months after the initial antigen injection, the rabbits were studied

Preparation of lymphoid cells Cell populations were obtained from the popliteal mesenteric, and tracheo bronchial lymph nodes and from the lymph of the efferent popliteal vessel and the thoracic duct

Cell suspensions were prepared from the lymph nodes by teasing followed by filtration through double gauze The efferent lymph vessel was cannulated near the hilus of the popliteal lymph gland and the thoracic duct in the upper part of mediastinum All cell suspensions were washed three times by centrifugation before use

Culture methods Cell populations from the lymph were cultured in test tubes and in dialysis tubings, as described in detail by *Nilsson* (1967 b, 1969)

The lymphoid cells were suspended in a culture medium consisting of 30 per cent autologous serum obtained before the first antigen injection and 70 per cent Parker 199 The cells were cultured in test tubes during 3 9 days with a cell concentration of 2 mill /ml In dialysis tubing the cell concentra tion was varied between 15 and 120 mill /ml, the culture time being 9 12 days After culture, the cells were washed three times by centrifugation and used for immunofluorescence studies

Studies on antibody synthesis For studies on *de novo antibody synthesis in vitro cell popula* tions from the lymph of the efferent popliteal vessel and the thoracic duct were cultured in dialysis tubings at a cell concentration of 60 mill /ml during nine days in a medium containing dl valine 1 C^{14} (The Radiochemical Centre, Amersham, England) with a specific activity of 33 9 C/M The radioactivity of the media was 1 85 μC/ml The culture media were studied with immunodiffusion in agar gel with Ouchterlony's micro method After wash ing and drying the agar slides were studied with stripping film autoradiography using an exposure time of 6 12 weeks For further details, see *Nilsson* (1967b, 1969)

Immunofluorescence methods Heat fixed slides were analysed for antibody containing cells with a double layer and a more sensitive quadruple-layer technique described in detail by *Nilsson* (1967a, 1969)

Double layer technique In the first step, human IgG was used, in the second step, fluorescein isothiocyanate (FITC) conjugated goat antiserum *v* human IgG (Microbiol Assoc, Bethesda, Maryland)

Quadruple layer technique In the first and third steps, human IgG was used The second step consisted of a high titred rabbit antiserum *v* human IgG In the fourth step FITC-conjugated goat antiserum *v* human IgG was used

Two types of control slides were made In type one only the diluent was used in the first step of the double layer and in the first and third steps of the quadruple layer technique, the other steps being as above In type two FITC conjugated goat antiserum *v* guinea pig serum globulin (Microbiol Assoc Bethesda Maryland) was used in the final step, the other steps being unchanged

RESULTS

Rabbits injected with 6 mg of antigen into the toe pads at day 0 and day 10 showed at day 14 an immune response with antibody production with practically no demonstrable

TABLE 1 *Immunofluorescence studies of Antibody Containing Cells in Lymph Nodes (l n) and the Lymph from the Efferent Popliteal Vessel and the Thoracic Duct*

Experimental group	Popliteal l n	Mesenteric l n	Tracheo-bronchial l n	Efferent lymph vessel	Thoracic duct
t p 6 μg	+ + 5/5			+1/5 + +1/5	0/2
t p 6 mg	+ + 44/45	0/4	+1/2	+7/14 + +1/40	0/3
t p & i v	+2/3 + +1/3	+2/3 + +1/3	+2/3 + +1/3		+1/3

55 rabbits immunized with human IgG according to three different schedules The fractions give the No of positive rabbits (numerator) in relation to the No of rabbits studied (denominator)
t p 6 μg and t p 6 mg = injected with 6 μg and 6 mg of antigen respectively, into the toe pads at day 0 and day 10 and studied at day 14
t p & i v = injected into the toe pads and intravenously over a period of 13 months
\+ = positive only with the sensitive quadruple layer technique
\+ + = positive also with the double layer technique

delayed skin response (*Nilsson* 1969) Immunofluorescence studies on such rabbits (see Table 1) showed that antibody containing cells were mainly found in the regional popliteal lymph node Positive cells were sometimes demonstrable in the efferent lymph cell population and exceptionally in distant lymph nodes but never in thoracic duct cell populations

It is possible by means of culture methods to demonstrate the presence of previously undetectable antibody synthesizing cells in a cell population Thus sensitized lymphoid cells may contain small quantities of antibody that cannot be detected by the present method However their antibody content might reach detectable levels after further maturation That this indeed is true was shown in studies on such cultures of cell populations from the efferent lymph vessel Using test tube cultures a conversion from a negative to a positive score of antibody containing cells *in vitro* occurred in 10 out of 18 experiments The cell populations from all 18 rabbits studied were negative before culture Furthermore a conversion of the remaining eight cell populations could have escaped detection as it could be demonstrated that a positive culture could again convert to a negative score when the culture time was prolonged Cell populations from six rabbits out of another seven cultured in dialysis tubings converted from a negative to a positive score Only four of the seven cell populations were cultured at optimum cell concentration (60 mill /ml) and all four converted to a positive score

Thoracic duct cell populations were studied in the same way, all with negative results Thus no conversion from a negative to a positive score was obtained either in test tube cultures (4 rabbits) or in cultures in dialysis tubings (4 rabbits)

Rabbits were therefore injected with 6 μg of antigen according to the same schedule to obtain an immune response with an augmented production of circulating antibodies but also a delayed type skin reactivity (*Nilsson* 1969) Apparently as shown in Table I no increased content of antibody-containing cells was demonstrable in cell populations either from the efferent lymph vessel or from the thoracic duct despite the augmented production of circulating antibodies in these rabbits

Finally, rabbits were injected into toe pads and intravenously over a period of 13 months Table I shows that this immunization schedule elicits a more generalized antigenic stimulation of the lymphoid tissue contributing to the cell population of the thoracic duct In agreement with this one of the three thoracic duct cell populations studied contained antibody containing cells One of the negative thoracic duct cell populations was cultured in dialysis tubings a conversion to a positive

score being obtained Thus the content of antibody containing cells in the thoracic duct lymph of rabbits immunized in this way was comparable with the content of such cells in the efferent lymph from the regional lymph node of rabbits studied at a time when a more localized antigenic stimulation of the lymphoid tissue could be expected

The method of incorporation of radioactively labelled amino acids into antibodies was used to study whether a conversion from a negative to a positive score of antibody containing cells during culture indicates a *de novo* synthesis of antibodies *in vitro* or merely an unmasking of antibodies previously synthesized *in vivo* Three cell populations from the efferent lymph vessel and one thoracic duct cell population all converting during culture, were studied Radioactively labelled antibodies were demonstrable in all four culture media (cf Fig 1) Apparently an increase in the score of antibody containing cells during culture was a sensitive, although indirect, proof of *de novo* synthesis of antibodies in vitro

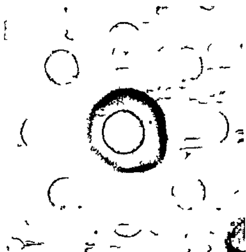


Fig 1 Autoradiogram of an immunodiffusion slide showing a labelled precipitate In the central well was placed concentrated culture medium in the peripheral wells dilutions of the antigen Thoracic duct cells obtained from a rabbit injected into the toe pads and intravenously over a period of 13 months were cultured in dialysis tubings for nine days at a cell concentration of 60 mill./ml with valine-C^{14} added to the culture medium

DISCUSSION

The results of the present study agree with the view that antibody synthesizing lymphoid cells spreading along the lymph route take part in the dissemination of the immune response Thus a *de novo* synthesis of antibodies *in vitro* was conclusively proved in cell populations from the efferent lymph vessel and the thoracic duct

However, the opinions in the literature about the antibody synthesizing capacity of thoracic duct cell populations are controversial (see *Nilsson* 1969) The discrepancy in results can be explained in terms of differences in the immune response at the day of investigation The present study showed that the release of antibody containing cells was correlated to the extent of the antigenic stimulation of the lymphoid tissue contributing to the thoracic duct cell population Antibody containing cells were demonstrable in the efferent lymph from the regional lymph node, but never in the thoracic duct lymph at an immune response with a more localized antigenic stimulation of the lymphoid tissue The absence of positive cells in the thoracic duct populations could be caused by a dilution in the thoracic duct of antibody containing cells from the efferent lymph vessel with numerous negative cells from for instance the gut Some quantitative but inconclusive observations supported this view (*Nilsson* 1969) In agreement with this such cells were found in the thoracic duct lymph at an immune response with a more generalized antigenic stimulation of the lymphoid tissue contributing to the thoracic duct cell population Furthermore the interval between a booster injection and the time of study seems to be another variable of importance in this respect *Hallander* & *Danielsson* (1962) obtained positive results only during the limited period of 2–4 days after the booster injection, which obviously corresponded to the period of rapid antibody production as studied by *Wesslen* (1952) One possible explanation of the low frequency

of positive results in the study of *Hulliger* & *Sorkin* (1965) could be that the study was performed 4–5 days after the booster injection

The costs of this investigation were defrayed by grants from the *Swedish Cancer Society* (project 108 K 68), the *Swedish Medical Research Council* (project B 69 12 X 579) and the *Swedish Multiple Sclerosis Foundation* This study summarizes and amplifies a thesis (*Nilsson* 1969)

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Acta path microbiol scand Section B 79, 539-544 1971

SEROLOGICAL CROSS-REACTIONS OF TANNED ERYTHROCYTES SENSITIZED WITH STAPHYLOCOCCAL ANTIGENS

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Staphylococcal extracts and isolated antigenic preparations have been used to sensitize tanned sheep erythrocytes (TSE) and indirect haemagglutination has been studied in cross reaction inhibition and absorption experiments The sera employed were whole sera against bacteria and specific sera against TSE sensitized with antigenic preparations It was found that indirect haemagglutination of TSE sensitized with a bacterial extract or an isolated antigenic material is complex and involves at least three determinants Two of the determinants may be ascribed to the peptide subunit and the third to the sugar chain of the mucopeptide Variations in these structures were indicated by the serological results, and the method may be of value in screening bacteria for similarities or differences in mucopeptide structures

Antigens sensitizing normal sheep erythrocytes (NSE) or tanned sheep erythrocytes (TSE) for agglutination in homologous and heterologous antisera have been demonstrated in extracts of staphylococci (21 19, 17, 15, 7 13 9 18) The NSE sensitizing antigen studied by *Rantz et al* (19) was of carbohydrate nature and it has been suggested that the ability to sensitize TSE may be due to the cell wall teichoic acid (13, 14 1)

In previous investigations (5, 18) it was found that the antigen of *Staphylococcus aureus* sensitizing NSE was probably of the Rantz type In addition a peptide unit was shown to be essential for sensitization of TSE, and that pure teichoic acid was inactive in this context Later studies (3, 4) showed that sensitization of TSE was due to the cell wall mucopeptide and that its sugar chain, as well as its peptide subunit, exhibits serological specificities

That cross reactions can be instructive sources of information, relating chemical constitution to immunological specificity, has been demonstrated in the case of several polysaccharide components of bacterial origin In the present study extracts and isolated antigens from strains showing TSE– sensitizing ability were used, in order to determine whether the serological cross reactions were due to one or more shared determinants Cross reaction and absorption experiments with sensitized TSE may also be expected to be of value in demonstrating similarities and

Received 7 xii 70

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differences in the mucopeptide structure of various bacteria

MATERIALS AND METHODS

Strains Three *Staph aureus* strains, Wood 46 (6), Cowan I (18), 263 (8), and two *Staph epidermidis* strains 3519 and 1268 (11) were used for preparation of antigens and extracts

Growth and harvesting The bacteria were grown for 18 hr on nutrient agar in Petri dishes of 14 cm diameter They were then harvested by scraping with a glass rod

Bacterial extracts Extracts were prepared by suspending the organisms from 10 Petri dishes (10 15 g wet weight) in 15 ml of phosphate buffered saline pH 7 2 incubating at 37°C for 3 hr and then centrifuging at 10 000 *g* for 30 min Finally, the supernatants were heated for 15 min at 65°C to destroy haemolytic activity

Isolated antigenic materials Polysaccharide A (poly A) from strain Wood 46 and polysaccharide 263 (poly 263) from strain 263 were prepared according to the methods of *Haukenes* (6) and *Hofstad* (8), respectively Both these polysaccharides consist of N acetylglucosaminylribitol teichoic acids and of small mucopeptide fragments The amino sugar is β linked in the teichoic acid of poly A whereas it is α linked in the teichoic acid of poly 263 (2) Polysaccharide 3519 (poly 3519) and polysaccharide 1268 (poly 1268) were prepared as described by *Losnegard & Oeding* (11) These polysaccharides also have mucopeptide fragments in addition to the teichoic acids In poly 3519 it is a ribitol teichoic acid with β linked and trace of α linked N- acetylglucosamine but in poly 1268 there is a glycerol teichoic acid (2 12) Crude protein A was prepared as previously described (5)

Immune sera Antisera were raised in rabbits given intravenous injections of formalinkilled bacteria (16) or TSF sensitized with either poly A poly 263 or crude protein A (18)

Serological methods The TSF were prepared by treating NSF with a 1 40 000 solution of tannic acid The sensitization was performed essentially as described by *Morse* (13) Usually 0 1 ml of packed TSF was sensitized with 0 1 mg of isolated antigenic material or with 1 ml of bacterial extract *The sera were diluted twofold with* buffered saline to a total volume of 0 5 ml To each tube was then added 0 1 ml of a 1 25 per cent solution of the sensitized cells After thorough mixing the tubes were incubated at 37°C for 30 min and then at room temperature overnight Haemagglutination titres were recorded before (pattern) and after gentle shaking

Antisera were absorbed by adding excess sensitized TSF to the serum diluted 1 10 and incubating at 37°C for 30 min Absorptions were continued until the sera failed to agglutinate fresh sensitized TSF

Inhibition of indirect haemagglutination was carried out as follows Serial twofold dilutions (0 25 ml) of the substance to be tested were made in saline and an equal volume of antiserum (diluted 8 times less than its haemagglutination titre) was added to each tube The mixtures were incubated at 37°C *for 2 hr then* at 4°C overnight and finally examined for ability to agglutinate sensitized TSF.

RESULTS

The haemagglutination titres of TSF sensitized with either bacterial extracts or isolated preparations are shown in Tables 1 and 2. Cross reactions were observed between all systems when extracts were used for sensitization (Table 1) When isolated antigens were used to sensitize TSE (Table 2) no reaction was observed between TSF poly A and antiserum 1268 TSE poly 1268 and antiserum Wood 46 and TSF poly 263 and antiserum Wood 46 TSF crude protein A agglutinated in all antisera Although great variations in titres were found the titres were usually lower when extracts were used to sensitize TSE It was also observed that repeatable results were obtained when TSF were sensitized with isolated antigens but different extracts of the same strain gave variable titres in the same antiserum

TSE poly A was further tested for agglutination in 16 other *Staph aureus* antisera All antisera agglutinated TSF poly A but the titres varied from 1 160 to 1/20 480

TABLE 1 *TSF Sensitized with Extracts Haemagglutination Titres (Reciprocal Values) in Homologous and Heterologous Antisera*

TSF sensitized with extract of strain	Antisera Wood 46	263	3519	1268	Cowan I
Wood 46	2 560	160	1 [illegible]	160	[illegible]
263	160	1 [illegible]	80	3[illegible]	3 [illegible]
3519	[illegible]	5[illegible]	1 2[illegible]	6[illegible]	6[illegible]
1268	4	[illegible]	40	[illegible]	3[illegible]
Cowan I	[illegible]	1 [illegible]	[illegible]	[illegible]	3[illegible]

TABLE 2 *TSE Sensitized with Isolated Antigens Haemagglutination Titres (Reciprocal Values) in Homologous and Heterologous Antisera*

TSE sensitized with	Antisera				
	Wood 46	263	3519	1268	Cowan I
Poly A	10 240	5 120	5 120	–	5 120
Poly 263		10 240	80	80	1 280
Poly 3519	5 120	5 120	10 240	160	10 240
Poly 1268	–	1 280	640	1 280	2 560
Crude prot A	160	80	1 280	320	10 240

TABLE 3 *Haemagglutination Inhibition*

Haemaggl systems		Inhibitors				
TSE sensitized with	Antisera	poly A	poly 263	poly 3519	poly 1268	Crude prot A
Poly A	Wood 46	—	+	—	+	—
–	263	—	+	—	+	+
–	3519	—	+	—	+	—
–	Cowan I	—	+	—	+	—
Poly 263	263	+	—	+	+	+
–	3519	—	—	—	+	—
–	1268	—	—	—	—	+
	Cowan I	—	—	—	+	—
Poly 3519	3519	+	+	—	+	+
–	Wood 46	+	+	—	+	—
–	263	—	+	—	+	+
	1268	+	—	—	—	+
	Cowan I	+	+	—	+	+
Poly 1268	1268	+	+	+	—	+
–	263	+	+	+	—	+
–	3519	—	—	—	—	—
–	Cowan I	+	+	+	—	+
Crude prot A	Cowan I	—	+	—	+	—
–	Wood 46	—	+	—	+	—
–	263	—	—	—	+	—
–	3519	+	+	—	+	—
–	1268	+	+	+	+	—

\+ Agglutination, i e no inhibition — No agglutination, i e inhibition

The specific antisera against TSE-poly A TSE-poly 263 and TSE crude protein A gave rather low haemagglutination titres with homologous antigens (1/80 to 1/160) Cross reactions were obtained with all isolated antigenic preparations but the titres in heterologous sera were reduced one to two steps compared to those in the homologous sera

The results of inhibition studies using the isolated antigen preparations are presented in Table 3 Each preparation gave complete inhibition of the systems in which it was used as sensitizing agent, but the effectivity of the preparations to inhibit heterologous systems was variable Some haemagglutinating systems were completely inhibited by small amounts (μg) of antigen, whereas other systems were only partly inhibited or required relatively large quantities (mg) of antigen for complete inhibition In spite of cross-

TABLE 4 *Haemagglutination in Sera Absorbed with TSE Sensitized with Bacterial Extracts*

Haemaggl systems		Absorbents				
TSE sensitized with extract of	Absorbed sera	TSE sensitized with extract of				
		Wood 46	263	3519	1268	Cowan I
Wood 46	Wood 46	–	+	–	+	–
–	263					
–	3519					
	1268		–			
	Cowan I	–	+		+	
263	263	+	–			
–	Wood 46					
–	3519	–	–	–	+	–
–	1268	–	–	–	–	–
–	Cowan I	+	–	+	+	–
3519	3519		+		+	+
–	Wood 46	–	+	–	+	+
–	263	–		–	+	–
–	1268				–	–
	Cowan I		+	–	+	–
1268	1268	+	+	+		+
	Wood 46		–	–	–	–
–	263		–	–	–	
	3519					–
	Cowan I	+				
Cowan I	Cowan I	+	+		+	
–	Wood 46		+	–	+	
–	263	+		+	+	–
	3519	+	+	–	+	–
	1268	–	+			–

\+ Haemagglutination No haemagglutination

reactions far from all systems were inhibited by other antigenic preparations than those used for sensitization An antigenic preparation did not always block all haemagglutinins in homologous antisera

All haemagglutinins in antiserum 1268 were blocked after addition of poly 263 together with crude protein A On the other hand, antiserum 263 still contained haemagglutinins against TSE-poly 263 after inhibition with a mixture of all antigenic preparations (equal proportions), except poly 263

The results of absorption experiments are shown in Table 4 All sera were absorbed with TSE sensitized with bacterial extracts and then examined for remaining haemagglutinins It was revealed that a serum absorbed with TSE sensitized with an extract of the homologous strain was not only exhausted for haemagglutinins against the absorbent but also for haemagglutinins against the other bacterial extracts Furthermore it appeared that several antigenic determinants were present in the extracts and that corresponding antibodies were in the antisera

Wood 46, 3519 and Cowan I antisera were not only exhausted by TSE sensitized with extracts of corresponding strains but *in most* cases with extracts of each of these three strains Antisera to the strains 263 and 1268 were completely absorbed only by TSE sensitized with extracts of homologous strains

DISCUSSION

These experiments show that the *indirect* haemagglutination of TSE sensitized with staphylococcal extracts or isolated antigenic preparations is rather complex Obviously the sera contain haemagglutinins of different

specificities and both the extracts and the antigenic preparations have various determinants The amount of each determinant may vary from one extract or preparation to another, as may also the amount of each antibody specificity in the sera, thus giving rise to variations in antibody titres Furthermore, an antigenic preparation may not always contain all the determinants of its strain participating in indirect haemagglutination of TSE The results of haemagglutination thus depend on (a) the relative amount of the different active groups present in the antigenic material and (b) the relative amount of corresponding antibodies in the antisera

The evaluation of the present results was complicated by some apparently contradictory reactions Most of these contradictions (e g lack of cross reaction between isolated poly A and poly 1268 in contrast to extracts of corresponding strains lack of agglutination of TSE poly 263 in antiserum Wood 46 (Tables 1 and 2) and complete absorption in some but not all sera of haemagglutinins against a certain extract (Table 4) are most likely due to (a) and/or (b) (see above)

Cross reactions between all strains and antisera (Table 1) demonstrate the presence of a least one common antigenic determinant, tentatively called D_1 All strains, except 1268 seem to have a second determinant (D_2) in common Another determinant (D_3) is apparently shared by Wood 46, Cowan I and 3519, but not by the other two strains Furthermore each of the strains 263 and 1268 most likely contains at least one specific determinant D_4 and D_5 respectively (Table 5) The strains Wood 46, 3519 and Cowan I seem to be very similar with respect to antigens capable of sensitizing TSE although the relative amount of each determinant apparently varies from strain to strain The Cowan I strain probably has small amounts of D_4 in addition to D_1 D and D_3 It is further evident that most *Staph aureus* strains share one or more of the determinants

Previous experiments (3, 4) demonstrated that sensitization of TSE was due to both the sugar chain and the peptide subunits of the mucopeptide This finding is in agreement with the results obtained by *Rolicka* & *Park* (20) in a study of mucopeptide preparations and their corresponding antibodies

TABLE 5 *Antigenic Determinants Active on Haemagglutination of Tanned Sheep Erythrocytes*

Strains	Determinants				
Wood 46	D_1	D_2	D_3		
Cowan I	D_1	D	D_3	(D_4)	
263	D_1	D_2		D_4	
3519	D_1	D	D_3		
1268	D_1				D_5

Since crude protein A contains no detectable sugars (5), the cross reaction between TSE crude protein A and TSE sensitized with polysaccharides (poly A, poly 263) in whole sera and specific antisera must be due to a common peptide determinant(s) In mucopeptides both the tetrapeptide and the cross linking bridge are serologically active (10), and accordingly it is tempting to assume that TSE crude protein A has two determinants (D_1 and D) The peptide subunits in the mucopeptides of strains Wood 46, Cowan I, 263, and 3519 should thus have identical structures Only one determinant (D_1) seems to be present in *Staph epidermidis* strain 1268 indicating a peptide structure differing in some respects from that of the other strains The determinants, D_3, D_4 and D_5 seem to be correlated to the sugar moieties of the mucopeptides Various antigenic specificities of the sugar chains may be results of differences in configuration or acetylation, or in the substituents of the sugars The configuration of aminosugars in the mucopeptide might be analogous with that in the teichoic acid

It seems possible, given a suitable reference system that indirect haemagglutination of TSE combined with absorption of sera might be a useful method for the screening of bacteria for structural similarities in the mucopeptide This might also be of taxonomical value

TABLE 4 *Haemagglutination in Sera Absorbed with TSF Sensitized with Bacterial Extracts*

Haemaggl systems		Absorbents				
TSE sensitized with extract of	Absorbed sera	Wood 46	TSE sensitized with extract of 263	3519	1268	Cowan I
Wood 46	Wood 46	—	+	—	+	–
–	263					
	3519					
	1268					
	Cowan I	—	+		+	
263	263	+				
–	Wood 46					
	3519		—	—	+	—
	1268	—	—	—	—	—
–	Cowan I	+	—	+		—
3519	3519		+		+	+
–	Wood 46	—	+	—	+	+
–	263		—	—	+	—
–	1268				—	—
	Cowan I		+		+	
1268	1268	+	+	+		
	Wood 46			—		
	263			—	—	—
	3519					
	Cowan I	+				
Cowan I	Cowan I		+			
–	Wood 46		+	—	–	—
	263	+		+	+	—
	3519	+	+	—	+	
	1268	—	+	—		–

+ Haemagglutination — No haemagglutination

reactions far from all systems were inhibited by other antigenic preparations than those used for sensitization An antigenic preparation did not always block all haemagglutinins in homologous antisera

All haemagglutinins in antiserum 1268 were blocked after addition of poly 263 together with crude protein A On the other hand antiserum 263 still contained haemagglutinins against TSF poly 263 after inhibition with a mixture of all antigenic preparations (equal proportions) except poly 263

The results of absorption experiments are shown in Table 4 All sera were absorbed with TSF sensitized with bacterial extracts and then examined for remaining haemagglutinins. It was revealed that a serum absorbed with TSE sensitized with an extract of the homologous strain was not only exhausted for haemagglutinins against the absorbent but also for haemagglutinins against the other bacterial extracts Furthermore it appeared that several antigenic determinants were present in the extracts and that corresponding antibodies were in the antisera

Wood 46 3519 and Cowan I antisera were not only exhausted by TSF sensitized with extracts of corresponding strains but in most cases with extracts of each of these three strains Antisera to the strains 263 and 1268 were completely absorbed only by TSF sensitized with extracts of homologous strains

DISCUSSION

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Acta path microbiol scand Section B 79 545–548 1971

TRIMETHOPRIM FOR THE PREVENT[illegible] OF OVERGROWTH BY SWARMING *PROTEUS* IN THE CULTIVATION OF GONOCOCCI

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Chief R Saxholm, M D

799 samples to be examined for gonococci by culture were inoculated onto a [illegible] medium which contained the concentration of vancomycin colistin and nystatin [illegible] employed and b) the same medium containing in addition 5 µg/ml trimethoprim [illegible] The routine cultures were, in 21 instances, overgrown by swarming *Proteus* In all [illegible] where trimethoprim was used, swarming was completely hindered whilst growth of gonococci was apparently unhampered A further 100 specimens, which on routine cul[illegible] showed growth of swarming *Proteus* were then inoculated onto medium [illegible] trimethoprim where swarming growth appeared only once and not until the second day of incubation Gonococci were isolated more often where trimethoprim was added than when routine medium was used The result was considered to be so favourable that trimethoprim is now added, as a matter of routine, to the medium used for isolation of gonococci at this laboratory Composition of this medium is given in the context

Cultivation of gonococci has often been difficult previously because other bacteria growing on the media have hidden or impeded the growth of gonococci Samples have to be taken from sites which are often heavily contaminated by other bacteria This is particularly the case with samples from the female genital tract and from the rectum *Thayer and Martin* (5, 6) introduced selective culture media first by adding ristocetin and polymyxin B and later replacing these antibiotics by vancomycin colistin and nystatin As a result the greater part of the contaminated growth is suppressed or inhibited which makes the isolation of the gonococci far easier As a rule, however the added antibiotics cannot hinder swarming of *Proteus*, and therefore the cultural isolation of gonococci is still frequently impeded

Trimethoprim is a comparatively new antibacterial substance It acts as an inhibitor of dihydrofolic acid reductase and has been used in combination with sulphonamides in the treatment of gonorrhoea by *Csonka and Knight* (1) with good results because of the marked potentiated antibacterial effect of the substances upon the gonococci When tested alone, however, trimethoprim has a remarkably slight effect upon gonococci, as shown *in vitro* by *Darrell and co-workers* (2) Thus, according to their investigations the inhibiting effect upon *Proteus vulgaris* and *mirabilis* is on an average at least [illegible] times as great as upon gonococci It [illegible]

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therefore, be an idea to add a suitable concentration of trimethoprim to culture media *in the hope that swarming of Proteus* would be checked, and yet the growth of the gonococcus remain unaffected *Seth* (4) has used trimethoprim lactate at a concentration of 8 µg/ml to brain heart infusion blood agar, plus the Thayer-Martin inhibitors This was found to suppress the growth of *Proteus* species without interfering with the growth of *N gonorrhoeae Riddel and Buck* (3) employed a concentration of 3 µg/ml trimethoprim in chocolate agar and also in saponin lysed blood agar These media also contained vancomycin and colistin and at first nystatin, later replaced by pimaricin Using clinical material, swarming of *Proteus* was completely suppressed and the recovery rate of the gonococcus was increased

At this laboratory we have tried different concentrations of trimethoprim lactate in different culture media, with and without the addition of vancomycin *and colistin* We have come to the conclusion that a concentration of 5 µg/ml trimethoprim lactate in our standard medium for culture of gonococci containing the Thayer-Martin combinations of antibiotics gave the most satisfactory results

The purpose of this paper is to present the results of cultivation of *N gonorrhoeae* with this medium

MATERIALS AND METHODS

Our routine culture medium (CVN medium) for the isolation of gonococci is as follows A basal medium consisting of Bacto Beef Extract (Difco) 1 g Proteose peptone (Oxoid code L 46) 10 g NaCl 5 g Agar Agar gereinigt (E Merck Darmstadt, art 1614) 12 g $Na_2HPO_4 \cdot 12H_2O$ 2 g aqua dest 1000 ml pH adjusted to 7 2 7 4 After being autoclaved for 15 minutes at 120°C the basal medium is stored in the refrigerator Before use the basal *medium is melted by steaming and* kept at 50°C Before pouring 70 ml defibrinated horse blood is added per litre vigorously shaken and placed in a 70-75°C water bath for approximately 30 minutes or until the medium has achieved the right brown colour The medium is cooled to approximately 50°C and the following ingredients are added per litre 10 ml of a solution corresponding to BBL's IsoVitalex enrichment (with the exception of p-aminobenzoic acid) vancomycin hydrochloride (Vancocin Intravenous Lilly), colistin methanesulphonate sodium (Co mycin, Lundbeck and Co) and nystatin suspension (Mycostatin sterile powder, Squibb), giving final concentrations for these 3 antibiotics of respectively 3 µg/ml 7 5 µg/ml and 10 units ml

The trimethoprim culture medium (CVN Trim medium) contained a 2 per cent solution of trimethoprim lactate (Burroughs Wellcome and Co) sterile filtered through Carlson Ford Filter FK and added to the medium at the same time as the above mentioned antibiotics giving a final trimethoprim concentration of 5 µg/ml The 5 ingredients had been kept in suitable dilutions in a frozen state at —20°C, with the exception of the nystatin suspension, which was kept in the refrigerator The poured plates were kept in the refrigerator and *then placed at 37°C* the day before they were used and were usually not more than 3 days old before they were employed

The first part of the investigation consisted of 799 specimens sent to the laboratory on charcoal swabs in solid Stuart's transport medium to be examined for gonococci There were 387 urethral 284 cervical 106 rectal and 22 vaginal specimens *The swabs were streaked* onto the culture media and further spread by platinum loops Each swab was used to inoculate one medium with and one medium without trimethoprim and the medium which was to be used first was alternated each day The cultures were incubated for 18 20 *hours* at 36 5°C in 5 per cent CO_2 atmosphere and whenever necessary a further 24 hours *at the same* temperature, but without CO The diagnostic criteria for a positive gonococcal culture were Gram negative diplococci growing in typical oxydase positive colonies no growth on ... agar acid from glucose but not from maltose and sucrose Swarming *Proteus* and overgrowth *of non* swarming microbes was recorded in addition to growth of gonococci

In the second part of the investigation the swabs were put back *into Stuart medium* after inoculation on the CVN medium and kept in the refrigerator until the next *day The swabs* which had caused swarming *Proteus on the CVN* medium were then inoculated ... CVN Trim medium ... all 100 specimens For ... the method was the same as that used in the ... part of the investigation

RESULTS

Comparison between ... tivation on medium with and medium without trimethoprim can be seen in Table 1 It may be noted that there is not a *single exampl*... swarming *Proteus*

TABLE 1 *Comparison of 799 Samples Cultured for Gonococci Simultaneously on Routine Medium Containing Colistin, Vancomycin and Nystatin (CVN medium) and on the Same Medium with the Addition of 5 μg Trimethoprim Lactate per ml (CVN Trim Medium)*

	CVN medium			CVN Trim medium		
	Days of incubation			Days of incubation		
	1	2	total	1	2	total
Confluent growth of swarming *Proteus*	9	12	21	0	0	0
Overgrowth by non swarming microbes	25	28	53	18	16	34
Gonococci grown	115	30	145	120	27	147

on CVN-Trim medium Overgrowth of other microbes was also less apparent on this medium as compared with CVN medium *Gonococci, however, were found in 147* instances on the CVN-Trim medium, as against 145 on the CVN medium, a gain of only two CVN-Trim medium showed growth of gonococci i 3 cases where CVN medium showed overgrowth of *Proteus,* but there was one case of growth of gonococci on the routine medium and not on the trimethoprim medium

The results from the specimens which were streaked onto CVN-Trim medium after first *showing growth of swarming Proteus on* CVN medium an be seen in Table 2 Among the 100 specimens on the CVN medium, 70 plates were completely overgrown after 1 day, whilst the rest took 2 days We were able to save 1 gonococcal culture on the routine medium before the plate was overgrown by *Proteus* There was no overgrowth of swarming *Proteus on the* CVN Trim medium after 18 20 hours incubation, but after 2 days one plate was overgrown by *Proteus* Four plates were overgrown by non swarming microbes after 1 day and two more after the second day From these 100 specimens we were able to isolate gonococci in 6 instances on the CVN Trim medium, whilst on the CVN medium gonococci were isolated only once

DISCUSSION

The anti bacterial effect of trimethoprim is not antagonized by p aminobenzoic acid like the sulphonamides, but other constituents in some culture media can interfere with trime-

TABLE 2 *Results of Inoculation on Culture Medium Containing Trimethoprim (CVN-Trim medium) of 100 Gonococcal Samples Which Had Shown Growth of Swarming Proteus on Routine Culture Medium (CVN Medium)*

	CVN medium			CVN Trim medium		
	Days of incubation			Days of incubation		
	1	2	total	1	2	total
Confluent growth of swarming *Proteus*	70	30	100	0	1	1
Overgrowth by *non-swarming* microbes	0	0	0	4	2	6
Gonococci grown	1	0	1	4	2	6

therefore, be an idea to add a suitable concentration of trimethoprim to culture media in the hope that swarming of *Proteus* would be checked, and yet the growth of the gonococcus remain unaffected *Seth* (4) has used trimethoprim lactate at a concentration of 8 µg/ml to brain heart infusion blood agar, plus the Thayer Martin inhibitors This was found to suppress the growth of *Proteus* species without interfering with the growth of *N gonorrhoeae* *Riddel and Buck* (3) employed a concentration of 3 µg/ml trimethoprim in chocolate agar and also in saponin lysed blood agar These media also contained vancomycin and colistin and at first nystatin, later replaced by pimaricin Using clinical material, swarming of *Proteus* was completely suppressed and the recovery rate of the gonococcus was increased

At this laboratory we have tried different concentrations of trimethoprim lactate in different culture media, with and without the addition of vancomycin and colistin We have come to the conclusion that a concentration of 5 µg/ml trimethoprim lactate in our standard medium for culture of gonococci containing the Thayer Martin combinations of antibiotics gave the most satisfactory results

The purpose of this paper is to present t[h]
results of cultivation of *N gonorrhoeae* [o]
this medium

MATERIALS AND METH

Our routine culture medium (CVN f
the isolation of gonococci is as foll
medium consisting of Bacto Beef E
1 g Proteose peptone (Oxoid co
NaCl 5 g Agar Agar gereinigt (F
stadt, art 1614) 12 g Na_2H
aqua dest 1000 ml pH adjuste
being autoclaved for 15 min
basal medium is stored in th
use the basal medium is me
kept at 50°C Before pouri
horse blood is added per
and placed in a 70 75°C
mately 30 minutes or
achieved the right brow
cooled to approximatel
ingredients are added p

corresponding to BBL s IsoVitalex er
(with the exception of p-aminobenzoic a
comycin hydrocloride (Vancocin 1
Lilly), colistin methanesulphonate so
mycin Lundbeck and Co) and nysta
(Mycostatin sterile powder Squibb
concentrations for these 3 antibiot
3 µg/ml 7 5 µg/ml and 10 units

The trimethoprim culture me
medium) contained a 2 per ce
thoprim lactate (Burroughs V
sterile filtered through Carls
added to the medium at
above mentioned antibiot
thoprim concentration of
ents had been kept in s
state at —20°C, with
suspension which w
poured plates were
then placed at 3
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GROUP R STREPTOCOCCI IN MAN

Group R Streptococci as Aetiological Agent in a Case of Purulent Meningitis

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A new case of human infection caused by group R streptococci (*de Moor* 1963) is reported Thus the total number of cases described in the literature is brought up to four

In 1963, *de Moor* (1) reported studies on streptococci isolated from infections in pigs The entrance of the infection is apparently unknown The initial stage was bacteriaemia which was followed by septicaemic complications Some cases might recover

These streptococci were designated groups R S and T Among 2000 strains isolated from monkeys cattle sheep horses dogs cats rabbits mice and guinea pigs none belonged to these groups

In 1968 appeared the first report on streptococci of group R causing infection in man namely two cases of meningitis and one fatal case of sepsis (*Perch et al* (2)) The group R streptococci isolated from man proved to be identical serologically as well as biochemically with the reference strain isolated from a pig Altogether 34 biological tests were made besides cross absorptions and precipitation tests One of the human strains occurred in the α form

To our knowledge no reports on group R streptococcal infections in man have been published since then Therefore we think it relevant to publish a short note on a further case of meningitis caused by such an organism

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MATERIAL AND METHODS

The strain (L J /69) to be accounted for here was isolated from the spinal fluid where it was present in great numbers The methods of the biochemical and serological investigations were described in detail in a previous paper (2)

RESULTS

Strain L J produced a soluble haemolysin and behaved completely like a group R streptococcus when subjected to the 34 biological tests accounted for earlier (2) It was resistant to sulphathiazole weakly sensitive to streptomycin strongly sensitive to penicillin tetracycline, chloramphenicol and erythromycin and almost resistant to bacitracin

No antiserum was prepared against strain L J but in absorption tests using antisera produced by strains of a pig and a patient (2) and organisms of strain L J no agglutinins and precipitins appeared to remain unabsorbed No Neufeld reaction could be obtained with these antisera after absorption

CASE HISTORY

J No 1822/69 A 68 year old farmer keeping pigs cattle and fowls On Nov 23rd 1969 severely ill with shivering Temperature recorded as 38 9° C in the morning of Nov 24th In the morning of

Nov 25th the patient was confused and immediately hospitalized On admission he was still in a confused and disoriented state, with nuchal rigidity and a temperature of 36 8° C The spinal fluid was turbid but not sanguineous Examination of the spinal fluid showed a bloodcell count of 149 erythrocytes/μl, 3685 leucocytes, 90 per cent polymorphonuclear, 10 per cent mononuclear, the protein level was 3 80 g/l Treatment with penicillin streptomycin and sulphathiazole was instituted

Direct microscopy of the spinal fluid revealed numerous Gram positive encapsulated diplococci which on isolation proved to be streptococci belonging to group R No growth of group R streptococci was obtained from blood or throat swabs After the finding of Gram positive encapsulated diplococci was established, therapy with penicillin and sulphathiazole was continued On Nov 27th the second day after admission, the patient regained normal consciousness Antibiotic therapy was continued for a week, and the patient was sent home three weeks after the date of admission

Asked in August 1970, his wife stated that her husband had not regained his former health, although he was still able to take care of his small farm

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Acknowledgements to Professor Viggo Faber M D (Blegdam Hospital Copenhagen), who put the patient s data at our disposal.

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TRANSFORMATION OF RABBIT BLOOD LEUCOCYTES WITH A BOVINE ENCEPHALITOGEN —TEMPORAL PATTERN OF REACTIVITY

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Peripheral blood leucocytes from rabbits immunized with encephalitogenic antigen of bovine origin transform *in vitro* under the influence of the antigen Transformation was registered as incorporation of tritiated thym dine A closer analysis showed that lymphoid cells which transform are present in the circulation mainly before the debut of the disease Similar findings have previously been made on cytotoxic lymphoid cells A possible explanation is that 'sensitized lymphocytes capable of reaction with the antigen are withdrawn from the circulation at the attack on the central nervous sysem The implications of these findings for the understanding of similar phenomena at multiple sclerosis are briefly discussed

Lymphocytes from individuals immunized against a certain antigen transform *in vitro* to DNA synthesizing blast cells when exposed to the antigen in question This reaction is best correlated with a delayed type immune response (11) and has also been used to investigate experimental autoimmune dis orders and possible human counterparts Experimental autoimmune encephalomyelitis (EAE) can be induced by injection of a basic protein that can be isolated from brain substance This protein can be used to study lymphocyte transformation phenomena at EAE *Dau* & *Peterson* (5) demonstrated transformation of cells from rats and guinea-pigs injected with encephalitogenic protein of human origin No disease developed in the rats but most guinea pigs showed clinical signs of encephalomyelitis In both species, a dose dependent stimulation of spleen cells and also of peripheral blood leucocytes was noticed after treatment with encephalitogenic antigen Blast cell transformation was measured by incorporation of H^3-thymidine The rats were studied 18–28 days after injection, the guinea pigs, when signs of overt disease appeared (11–17 days after injection) or at the end of the third week if no clinical encephalomyelitis developed

A more recent study (13) also showed transformation of lymph node cells from guinea pigs with EAE when stimulated *in vitro* with brain antigen A crude antigen preparation was used and quantitation occurred with autoradiography after incorporation of tritiated thymidine Values obtained at day 18 were slightly—but not statistically significantly—higher than values at day 10 and 25 There was no statistically significant correlation between transformation rate and severity of the disease

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Dau & Peterson (6) used this method also to study multiple sclerosis in man, a disease repeatedly suggested as having autoimmune components. This experiment had been tried by some other authors (1, 4, 8), although with inconclusive results. *Dau and Peterson*, however, demonstrated a clear-cut and statistically significant better stimulation of leucocytes from MS patients than of leucocytes from controls, including some patients with other neurological diseases. More clear-cut results were obtained after partial purification of the leucocytes. Not all MS patients showed a response, and the authors state that the nine highest values of response were seen in patients whose disease—which they had had for a relatively short time—tended to be exacerbated.

Berg & Källen (3) demonstrated that the peripheral blood of patients with multiple sclerosis contains lymphocytes that attack and destroy neuroglia cells in tissue culture in a way similar to that described in the case of animals with EAE (2, 9, 10). Not all MS patients showed this characteristic, but it was found in patients with an active state of the disease. In EAE it was demonstrated (2) that such "aggressive" cells were only present during certain stages of the disease and usually disappeared soon after the debut of clinical signs of the disease.

These observations suggest a study of the temporal conditions in transformation reactivity of peripheral blood cells in animals injected with encephalitogenic protein to provoke EAE.

MATERIALS AND METHODS

Encephalitogenic antigen preparation. The encephalitogenic protein was prepared according to *Eylar et al.* (7) with some modifications. Briefly bovine brain tissue was defatted with chloroform methanol (2:1) and acetone and proteins were extracted from the defatted tissue with HCl at pH 2. The protein extract was neutralized with 10 per cent NH_4OH and pH 7 soluble proteins were precipitated with 80 per cent ammonium sulphate, dissolved in water and dialysed against water for 24 hours. The protein solution was twice passed through columns of DEAE-cellulose equilibrated with distilled water, and lyophilized. Finally the encephalitogenic protein was purified by gel filtration on a column of Sephadex G 100 superfine (5×86 cm) in 0.25 M ammonium acetate buffer pH 5.0.

When examined by electrophoresis in 15 per cent polyacrylamid gel according to *Reufeld et al.* (12) the protein preparation showed two minor faster moving contaminants, probably proteolytic fragments of the native protein. Amino acid analyses of the preparation gave values in good agreement with published data. Details of the antigen preparation will be reported (3 a).

Animals. Random bred rabbits obtained from a local dealer, and weighing 2–3 kg were used. Antigen injections were given only on day 0 in the toe pads of the hind legs. Antigen solution was mixed with an equal volume of Freund's complete adjuvant (*Difco*) and emulsified until drops of the emulsion did not float out when tested on water. Each rabbit was given three injections into different pads, each injection had a volume of 0.05 ml and contained 50 µg of antigen. 17 rabbits were thus injected. A further 5 were similarly treated, but injection of Freund's adjuvant emulsified with saline was used instead.

Leucocyte cultures. Peripheral blood leucocytes were cultured in test tubes containing 1.5 ml of a medium composed of 70 per cent Parker 199, 30 per cent normal rabbit serum (pooled), heparin and antibiotics.

In some experiments leucocytes were separated from erythrocytes by sedimentation using 6 per cent dextran (Macrodex Pharmacia). Roughly equal parts of dextran and heparinized blood were mixed and the mixture was left to sediment in a slanting tube for about one hour. Separation of RBC and WBC was only partial. Cell counts (nucleated cells) were adjusted to 2×10^6 cells per ml of culture medium.

In other experiments a micro method was used for culture. With a heparinized syringe approximately 1 ml of blood was drawn from an ear vein of the rabbit. A 12 gauge needle was used to put into each culture tube four drops of blood which was thoroughly mixed with the medium. These cultures gave consistent results equal to those prepared after cell counts as described above.

To each culture tube was added either 0.15 ml of encephalitogenic protein dissolved in saline at a concentration of 450 µg/ml or the corresponding amount of saline without the antigen [illegible]. In each group three parallel tubes were prepared.

Incubation took place at 37°C for 7 days. The cultures were labelled for one hour with 1 µC/ml of methyl H^3 thymidine (H^3 TdR) (Schwarz Bio Research Inc., spec. act. [illegible] C/mM). The cells were then spun down, washed once with Parker 199, extracted for 30 minutes with [illegible] 5 per

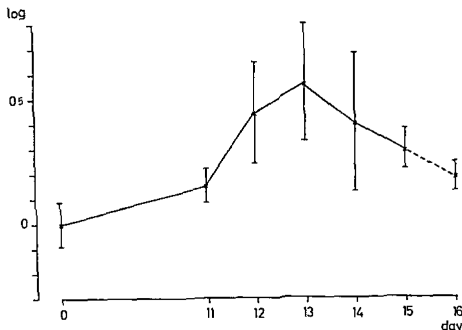


Fig 1 The effect of antigen on *in vitro* transformation of blood leucocytes from rabbits injected on day 0 with bovine encephalitogen in complete adjuvant log values show differences in log c p m of antigen containing cultures and log c p m of control cultures Means and standard errors of the mean are given

cent trichloracetic acid (TCA) and washed once with 5 per cent TCA and once with absolute alcohol The cell residue was dissolved in 0 6 ml of 1 N sodium hydroxide 0 1 ml of the dissolved material was mixed with 1 ml of Soluene and with 14 ml of scintillation fluid (300 mg dimethyl POPOP 5 mg PPO, 1000 ml toluene) The radio activity was assayed in a Packard TriCarb 3310 liquid scintillator and expressed as c p m Back ground activity was determined from a blank and was subtracted from the registered c p m Count ing was made during two 10 minute periods For the statistical treatment of the data, log c p m values were used The difference in response in the presence and absence of antigen was thus expressed as the difference in log c p m in anti gen-containing cultures and control cultures

RESULTS

The effect of antigen addition on H^3-TdR uptake at different days after immunization was first studied in eight rabbits Before the start of the ... effe... 1 sh...

c p m in antigen-treated cultures and in control cultures is very close to O In four of the rabbits, repeated tests were performed on days 11, 12, 13, 14, and 15 The mean differences between antigen-treated and control cultures at different days are seen in Figure 1 The other four rabbits were used for a determination on day 16 Also the mean value for the differences in these four rabbits is shown in Figure 1

The graph shows a definite stimulating effect of antigen, which reaches a maximum at day 13 with a mean log difference of 0 55 This value thus means that, on an average, the antigen-containing cultures show 3 5 times more H^3-TdR incorporation than cultures without antigen Furthermore, there is a definite decline of mean stimulating activity up to day 16

Figure 1 also shows that the standard errors of the means are definitely higher around days 12 14 than at other days, including day 0 This impression is further support-

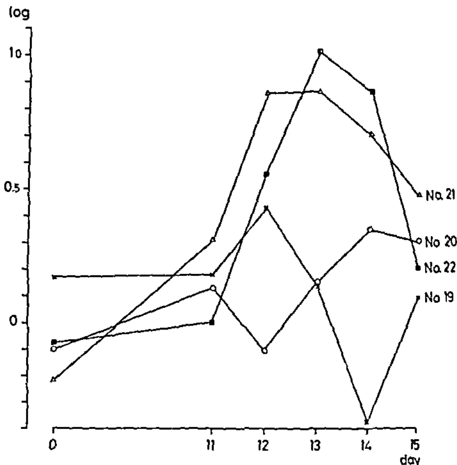


Fig 2 Diagram as in Figure 1 but showing values for each of four rabbits studied on days 0, 11, 12, 13, 14, and 15 Each graph is identified with the No of the rabbit

ed by a comparison of the registered variances using Bartlett's test This gives $\chi^2 = 12.7$ at 6 d f, $P < 0.05$ This could be due to different responses in the different rabbits to antigen stimulation at different days For this reason separate graphs were constructed for each of the four rabbits that were studied between days 11 and 15 Fig 2 shows the results

The graphs of rabbits No 21 and 22 resemble each other Both reach a very high level of response on about day 13 with a log difference of 0.88 and 1.03 resp that is 7.6 and 10.7 times higher thymidine uptake in the antigen-containing cultures Both decrease, but have not reached zero at the end of the experiment Neither of these two rabbits showed any clinical signs of encephalomyelitis during the 15 days but both had marked encephalitis when killed on day 18

Rabbit No 19 shows an early but not very marked increase of response up to a maximum at day 12 with a log difference of 0.44—that is 2.8 times increased thymidine incorporation From then on, a marked decrease occurs—even to negative values This animal had a fully-developed disease at day 14 Rabbit No 20 finally shows a slow increase of stimulation up to day 15, and no definite decrease was registered There were no clinical or histological signs of encephalomyelitis in this animal when it was killed on day 18

These observations indicate that cells which can be stimulated to blast transformation are found in the blood a few days before the beginning of the clinical disease, but that they disappear around the debut of the disease To obtain further support for this concept

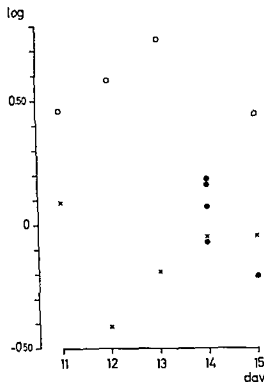


Fig 3 Diagram as in Figure 1 showing results based on 14 different rabbits × – rabbits injected with complete adjuvant only ○ – rabbits injected with antigen and adjuvant but not diseased at the time of determination ● rabbits injected with antigen and adjuvant and diseased at the time of determination

another series of rabbits was studied Nine rabbits were injected with the encephalitogenic antigen as described above and five rabbits were given Freund's complete adjuvant with saline instead of antigen All rabbits were killed and bled by heart puncture, and cultures were set up after separation of white blood cells Each animal was thus only studied once and the clinical and histological signs of encephalomyelitis were judged on the same day as the cells were removed The animals were killed on day 11 15 The results are shown in Figure 3

Among the nine animals injected with the encephalitogenic protein four showed a marked higher H^3 TdR incorporation in the presence of antigen in the culture—none showed signs of the disease at the time of death The other five rabbits all had definite encephalomyelitis, and their values scattered around zero The following mean values are obtained, given with the standard errors of the means

adjuvant injected controls —0 122±0 085
healthy animals injected with antigen
0 555±0 069
diseased animals injected with antigen
0 006±0 067

These values support the findings, presented above, that cells which can be stimulated by antigen to DNA synthesis can be demonstrated before the initiation of the disease but not when encephalomyelitis has set in

DISCUSSION

The data presented above confirm the observations of *Dau & Peterson* (5) that lymphoid cells from animals injected with encephalitogenic protein in complete adjuvant transform in culture when exposed to the antigen in question *Dau and Peterson* used human encephalitogen and guinea pigs and rats as experimental animals In the present study, bovine protein and rabbits were used Another difference is that most of the data presented by *Dau & Peterson* concern spleen cell cultures whereas our data refer to lymphoid cells from the peripheral blood *Dau & Peterson* present similar data in one table Guinea pig leucocytes from peripheral blood were shown to be stimulated by the antigen—the amount of the response in seven animals varied immensely from 1 3 to 80 1 times The clinical state of these animals was not given Referring to spleen cultures the authors state 'There was no correlation between *in vitro* response and the occurrence of clinical disease In fact the greatest *in vitro* response was from an animal without clinical disease

In our series of rabbit experiments, a temporal relation appeared between disease and *in vitro* transformation A definite decrease occurs in transformation activity in cultures from peripheral blood before the debut of the disease This phenomenon thus

resembles that found earlier concerning cyto toxic activity of lymphoid cells (2) A possible explanation is a disappearance of 'sensitized cells from the blood circulation at the attack on the central nervous system resulting in overt disease Such a phenomenon should be less conspicuous when spleen or lymph gland cells are studied

A parallel to human disease is possible When lymphoid cell activity is studied in human subjects, circulating lymphoid cells from the peripheral blood is practically always used In patients with multiple sclerosis, cells which transform when exposed to encephali togenic antigen (6) or cells which show a cytotoxic activity on neuroglia cell cultures (3) are *not found in all patients*, they are found more often in patients showing an active phase of the disease than in 'burnt out cases However, there is a poor correla tion with actual clinical relapse These obser vations would fit well with the animal models, we could expect only a certain per centage of individuals with the disease in an active state to have antigen reactive lymphoid cells in their circulation

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INHIBITION OF TRANSFORMATION IN *NEISSERIA MENINGITIDIS* BY ACRIFLAVIN AND ETHIDIUM BROMIDE

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Ethidium bromide and acriflavin at low concentration were found to inhibit transformation in *Neisseria meningitidis* The transformation efficiency gradually decreased with increasing concentrations of the drugs In the presence of RNA the effect of the drugs was reduced The uptake of ^3H labelled DNA by the recipient cells was also affected, although not as strongly as the transformation efficiency This shows that *N meningitidis* lacks a step in the DNA uptake that is insensitive to these drugs

In two strains of *Neisseria meningitidis* more thoroughly studied (M1 and Ne15), competence in the DNA mediated transformation has been found to depend upon a heritable factor called *cp* (5, 7) The behaviour of this genetic factor shows several features that are generally ascribed to episomal structures (5, 9) Many determinants of antibiotic resistance in *Enterobacteriaceae* and in *Staphylococcus* which have been shown to belong to extrachromosomal elements can be eliminated by ethidium bromide, or by acridine dyes (2, 4) Ethidium bromide and acriflavin have been found to be very weak general mutagens when acting on suspensions of meningococci, and both drugs seem preferentially to mutagenize at the replication point of the chromosome But in spite of the very weak mutagenic activity, ethidium bromide was found to be a powerful agent in eliminating competence in genetically competent (cp^+) variants of the two meningococcal strains One strain (Ne15) could also be rendered genetically incompetent by acriflavin at low frequencies (8)

In the *Haemophilus influenzae* system the transforming activity of ultraviolet light irradiated donor DNA is diminished in the presence of acriflavin, indicating that the repair mechanism may be inhibited by acriflavin (19) When acriflavin is added to competent cells of *B subtilis* the number of infectious centers produced by phage DNA is decreased (18) *Ayad* (2) found that the relative transforming activity in the *B subtilis* transformation system decreased with increasing acriflavin concentration Acriflavin, however, did not interfere with the uptake of transforming DNA, i e with the step leading to DNase insensitivity The reduction of the transforming activity to a negligible value seemed to be entirely due to an inhibition by acriflavin of the integration of the donor DNA into the recipient genome

In *N meningitidis* the step in the transformation leading to DNase insensitivity seems to be specific for homologous DNA (10, 11)

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This is in striking contrast to the findings from *B subtilis* (3, 20) and pneumococcus (14) It seems that competent *N meningitidis* cells lack the DNase insensitive sites outside the membrane suggested for *B subtilis* (1, 13), as well as the single stranded stage found in pneumococcus (14) These steps in the transformation of *B subtilis* and pneumococcus are obviously non specific in the sense that any double-stranded DNA of proper molecular size may participate (14 20)

The present paper reports the effects of ethidium bromide and acriflavin on the transformation efficiency and the uptake of transforming DNA in *N meningitidis* The experiments were undertaken to test the hypothesis that these chemicals in *N meningitidis*, unlike the situation in *B subtilis* (2) would inhibit the uptake as well as the transformation efficiency

MATERIALS AND METHODS

Strains Meningococcal strains were the wild type Strain M1 and Strain Ne15 both of Group B (8) Auxotrophic mutants as well as the streptomycin resistant mutants used for the production of transforming DNA were the same as have previously been used (8)

Media Blood agar plates and Heart Infusion Broth (HIB Difco) agar plates were used as solid complete media Fluid complete medium was Brain Heart Infusion Broth (BH Difco) The basal media have been described before (12)

Inhibition of transformation The inhibition of transformation by ethidium bromide or acriflavin was tested in a transforming system containing 1 4 ml HIB with 0 005 M CaCl 0 2 ml receptor cell suspension in saline 0 1 ml transforming DNA in SSC (NaCl citrate buffer 0 15 M NaCl + 0 015 M Na_2 citrate pH 7 4) 0 2 ml of dilution of the inhibitor to be tested The receptor cell culture was inoculated from an overnight culture on blood agar and grown in BH until the logarithmic growth phase (Absorbance 0 2 0 4) The cells were harvested in the centrifuge resuspended in saline, and inoculated into the transforming system. After incubation at 37°C with shaking for 20 min the inhibitor and DNA were added Transformation was terminated after the designed time, usually 30 min by the addition of 0 1 ml DNase giving a final concentration of 50 µg ml (11)

Inhibition of DNA uptake Inhibition of the DNA uptake by ethidium bromide or acriflavin was tested in a transformation system consisting of (10) 70 ml HIB with 0 005 M CaCl 10 ml HIB containing 180 µg ³H DNA plus the inhibitor to be tested, 10 ml receptor cell culture in BH The receptor culture was grown to logarithmic growth (Absorbance 0 5 0 6), inoculated into HIB plus CaCl, and after shaking for 20 min at 37° C the inhibitor plus DNA were added Transformation took place at 37° C with shaking and was terminated by the addition of DNase to a final concentration of 50 µg/ml After 5 min samples were taken for determination of viable counts and numbers of transformants After rapid chilling in ice salt, the nucleic acids were extracted from the cells as previously described and analysed by density gradient centrifugation on alkaline CsCl gradients (10)

Preparation of DNA Transforming DNA was either prepared by a modification of the Marmur procedure ('Crude DNA') (11), or by a more complicated purification ('Purified DNA') as previously described (10) ³H labelled DNA was prepared after labelling with ³H adenine (10)

RESULTS

Effect on the transformation efficiency The effect of ethidium bromide on the transformation system in *N meningitidis* was studied by incubating competent cells at 37°C with transforming DNA and various concentrations of the chemical The residual transforming activity gradually decreased with increasing ethidium bromide concentration (Fig 1) The concentration of ethidium bromide could not be increased much above 10×10⁻⁶M due to a comparatively high killing of the recipient cells particularly those of the Strain M1.

Fig 1 shows that acriflavin also reduces the number of transformants obtained in *N meningitidis* It seems that the drug is affecting the transformation efficiency in very much the same way as it does in the transformation of *B subtilis* (2)

It was observed that the inhibition of the transformation efficiency by ethidium bromide and acriflavin varied with the transforming DNA used The inhibition was always more pronounced when more purified DNA was used than in experiments with crude preparations of transforming DNA (Table 1) The latter preparations contain rather large amounts of RNA (11) It was indeed found that the addition of RNA

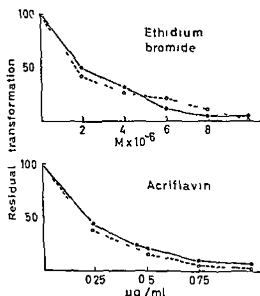


Fig 1 Effect of ethidium bromide and acriflavin concentration on the *V meningitidis* transformation system Competent cells (Ne15 or Ml 8 *arg his*) were incubated with DNA (Crude DNA) from Ml Str r (final concentration 2 5 μg/ml) for 30 min followed by DNase treatment The results are expressed as the transformation efficiency (T/E) at a particular drug concentration relative to that at zero concentration (—— 8 *arg his* - - - Ne15)

(from yeast) reduced the effect, particularly that of ethidium bromide, significantly

It is known that acridine (16, 17) as well as ethidium bromide (15) reacts with DNA The possibility had therefore to be considered that the effect on the transformation was due to an alteration of the transforming DNA The experiments indicate (Table 1) that preincubation of DNA with the inhibitors results in a slight increment in the inhibiting effect On the other hand, it was found that a major part of the transforming activity can be restored by dialyzing acriflavin or ethidium bromide treated DNA This suggests that the main effect on the transformation can only be obtained when the chemicals are present during the transformation process This is in agreement with the findings reported for the action of acriflavin on the *B subtilis* transformation system (2)

Effect on the uptake of DNA The influence of ethidium bromide and acriflavin on the uptake of ^3H labelled DNA has been shown in Table 2 Increasing concentrations of the chemicals resulted in a pronounced reduction of the specific labelling of the

TABLE 1 *Effect of the DNA Used and of RNA on the Inhibition by Ethidium Bromide and Acriflavin*

DNA used in transformation	Relative transforming efficiency per cent*	
	With ethidium bromide 6×10^{-6} M	With acriflavin 0 45 μg/ml
Crude DNA§	17 8	22 7
Purified DNA†	4 9	20 4
Purified DNA + RNA 50 μg/ml	23 4	25 8
Crude DNA preincubated with inhibitor for 30 min before the transformation	15 6	19 3
Crude DNA preincubated with inhibitor for 30 min and dialysed No inhibitor added during the transformation°	87	67

* T... (... ressed as recidual transforming activity

(11)

... s extracted and purified by treatment with pronase and RNase (10)

° Transforming DNA (50 μg/ml) was treated with the chemical and dialysed for 48 h against four changes of SSC

TABLE 2 *Effect of Ethidium Bromide and Acriflavin on the DNA Uptake and Transformation Efficiency in* Neisseria meningitidis

Expt no	Inhibitor added	Concentration of inhibitor	Transformation efficiency		Specific label of DNA	
			T/E	per cent	μg ^{3}H DNA/ μg total DNA	per cent
1	None	–	3.6×10^{-4}	100	7.06×10^{-4}	100
1	Ethidium Bromide	6×10^{-6} M	7.5×10^{-6}	2.1	4.43×10^{-4}	62.8
1	Ethidium Bromide	9×10^{-6} M	1.3×10^{-7}	0.04	2.52×10^{-4}	35.7
2	None	–	3.8×10^{-4}	100	4.50×10^{-4}	100
2	Acriflavin	0.2 μg/ml	1.8×10^{-4}	46.2	3.05×10^{-4}	67.8
2	Acriflavin	0.4 μg/ml	7.5×10^{-5}	19.8	1.14×10^{-4}	25.3

Transformation of M1 8 *arg his cp*⁺ with ^{3}H labelled Str r DNA for 30 min
Reisolation of DNA, and density gradient analysis as described in Methods

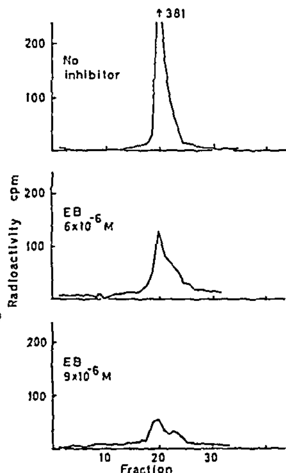


Fig 2 Effect of ethidium bromide (EB) on the uptake of transforming DNA during transformation of *N meningitidis* CsCl density gradients of extracts from cells (8 *arg his cp*) transformed with ^{3}H labelled *Str r* DNA

DNA peak When fairly high concentrations were used of the inhibitors there also seemed to occur a broadening of the radioactive DNA peaks, often with rather pronounced irregularity of the distribution (Fig 2 Fig 3) No single stranded DNA could be found in these experiments But it was also constantly found that the reduction in the transformation efficiency exceeded the reduction in the specific labelling of the DNA peak

DISCUSSION

At low concentrations acridine molecules are inserted or intercalated between adjacent base pairs in the DNA resulting in the extention and unwinding of the phosphodiester backbone (16, 17) It has also been shown that ethidium bromide binds to RNA and DNA and like acridines ethidium bromide is intercalated between adjacent base pairs of DNA (15) It seems that this type of alteration of the DNA structure can prevent its integration into the recipient genome of the competent cells of *B subtilis* (2)

The present experiments show that acriflavin as well as ethidium bromide also reduces the incorporation of transforming DNA into the recipient genome of competent meningococci, resulting in a reduced transformation efficiency

In *B subtilis* the addition of acriflavin does

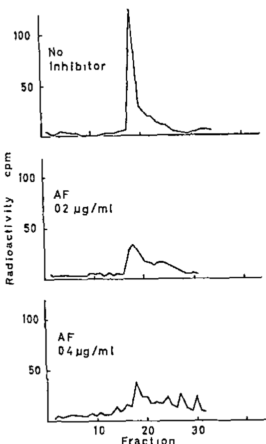


Fig 3 Effect of acriflavin (AF) on the uptake of transforming DNA during transformation of *N meningitidis* CsCl density gradients of extracts from cells (8 *arg his cp*) transformed with ^{3}H labelled Str r DNA

not interfere with the uptake of labelled DNA by the recipient cells, although the transformation efficiency is reduced to an almost negligible value (2) This is in striking contrast to the present experiments with *N meningitidis* which show that acriflavin as well as ethidium bromide strongly reduces the uptake of DNA These findings emphasize the previous observations that uptake of DNA by competent meningococci is distinct from the uptake by *B subtilis* cells as well as by pneumococci (10)

We have hypothesized that *N meningitidis* cells may lack the DNase insensitive sites outside the membrane suggested for *B subtilis* (1 13) Another feature of this model is that integration and penetration of the membrane represent the same step in the *N meningitidis* transformation, and that the uptake takes place at the replication point of the chromosome (6, 10, 11)

The present experiments give no unequivocal test of this model however, because there are two observations that are liable to alternative interpretations In the first place, the transformation efficiency in the presence of ethidium bromide or acriflavin is more reduced than the uptake of DNA Secondly, the density gradient profiles of the DNA taken up in the presence of the dugs seem to be a little distorted indicating that the radioactive DNA may in some way be abnormal When considering these observations it should be emphasized that the measurement of DNA uptake is based on the uptake by all cells present, also those which eventually turn out to be non viable, whereas transformation efficiency is calculated from the viable fraction of the cells It could thus be, that uptake and integration indeed occur simultaneously in *N meningitidis*, and that the 'abnormal DNA represents hybrids of donor and recipient DNA formed in the presence of the chemicals, which due to the drug action are largely non functional, and can not result in a transformed phenotype But the findings could also mean that uptake and integration are separate steps even in *N meningitidis*, and that both are inhibited by ethidium bromide and acriflavine, although not to the same extent The 'abnormal DNA could then be donor DNA molecules which have reacted with the drugs, and as a consequence can not be integrated, whereas they can still be taken up Further experimentation by means of heavy donor DNA will be necessary to find out whether the radioactive DNA represents hybrid DNA or donor DNA

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that protein contributes to the material in the gradient that gives absorption at 260 mμ when crude extracts are analysed and even in the area of the DNA peak. It would seem that the RNA present does not significantly interfere with the analysis of the DNA peak. It was subsequently assumed that the deproteinization steps give extracts suitable for gradient analysis of DNA under the circumstances.

In a few instances spurious bands were observed in density gradients from extracts not sufficiently purified such as also described by *Lacks et al.* (13). These bands often occurred in only one tube of two parallels run in the centrifuge.

The specific labelling of the double-stranded DNA peak was found to increase with time, and seemed to be proportional to the transformation efficiency (Fig. 4).

Lysates from genetically incompetent variants of the *N. meningitidis* Strain M1 (7, 8, 9) were also examined after exposure to ^{3}H DNA. In these cells no significant transformation takes place. The gradients showed an extremely small accumulation of radioactivity in the area of doublestranded DNA (Fig. 5). This bulk is too small to be observed in the previous experiments with mixtures of ^{32}P labelled DNA and RNA due to

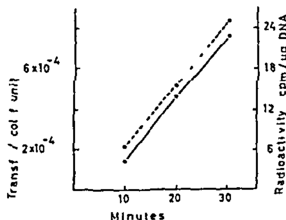


Fig. 4. Transformation efficiency and specific labelling of the DNA peak in density gradients as a function of duration of exposure of the recipient cells to ^{3}H DNA. (—— Transformation. - - - - Radioactivity.)

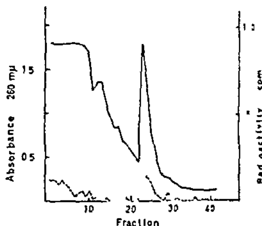


Fig. 5. Fate of homologous DNA in non-transformed *N. meningitidis* cells. Density gradients (pH 11) of extracts from cells (M1 8 *hu arg ep*) exposed to ^{3}H DNA from M1 Str-r (specific activity 7.0 × 10^3 cpm/μg) for 30 min. Specific labelling of the DNA peak is 3 × 10^{-3} μg ^{3}H DNA per μg total DNA. (—— Absorbance. - - - - Radioactivity.)

"noise" from the background and the radioactive RNA (Fig. 2).

When competent cells of *N. meningitidis* are exposed to ^{3}H labelled DNA from *E. coli* no significant radioactivity may be recovered in the fractions corresponding to DNA. But even when DNA specifically labelled with ^{3}H thymine (9) is used a very faint background activity is observed (Fig. 6).

Search for single-stranded DNA. Many attempts were made to demonstrate an appearance of radioactivity corresponding to single-stranded DNA during transformation of *N. meningitidis*. The experiments which included the exposure to labelled DNA for different times between 1 min and 45 min revealed no peaks in the fractions typical for denatured DNA.

The absence of single-stranded DNA in meningococcal extracts might conceivably be due to break down or to loss during the extraction procedure. Therefore denatured DNA was added to suspensions of transformed cells after the DNase treatment and washing but before the lysis of the cells. The DNA added seemed to be nearly quantitatively recovered after the extraction procedure (Fig. 7).

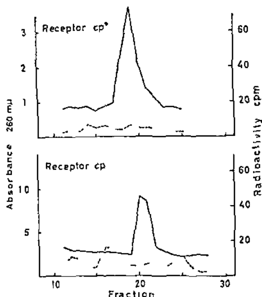


Fig 6 *Fate of heterologous DNA in N meningitidis* cells Density gradients (pH 11) of extracts from competent (Ml 8 *his arg cp*) and incompetent (Ml 8 *his arg cp*) cells exposed to ^{3}H thymine labelled DNA from *E coli* K12 (10) with specific activity 7 3 × 10^3 cpm/μg Time of exposure 30 min (—— = Absorbance – – = Radio activity)

DISCUSSION

Competent as well as incompetent meningo cocci retain tracer from mixtures of labelled nucleic acids probably by a binding to the surface (9) The finding that radioactive material sedimenting like RNA in the ultra centrifuge may be extracted from both types of cells indicates that a major part of this non-specifically adsorbed material may be RNA

The specific labelling of RNA decreases a little with time implying that the material bound to the cells is broken down This should probably be expected since it has been observed that RNA supports the growth of meningococci (23) We may ask whether significant amounts of radioactive material from RNA appears in DNA during the time of incubation According to the experiments performed with incompetent cells and with heterologous nucleic acids it would seem that this is not so

Binding of RNA in relatively large quantities has not been reported from other transformable species In pneumococcus it would appear that competent cells cannot indiscriminately retain large molecules In particular RNA is bound at most 1 per cent as strongly as DNA, if at all (14) We do not know why RNA is relatively strongly bound by meningococci But since RNA is bound to basic proteins (5) the reason could be a difference between the surface proteins of the transformable species

Since RNA seems to be bound by the cells it becomes of importance to know whether these molecules may interfere with the DNA transformation The inhibition experiments previously reported (9) indicate that this is not so, and that the DNA targets on the bacterial surface may be different from the sites to which RNA is bound

But other material than RNA is also bound non specifically to the cells, and at least some

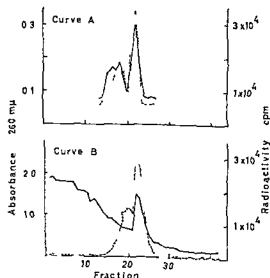


Fig 7 Separation of native and denaturated DNA from *N meningitidis* by density gradient centrifugation (pH 11) *Curve A* Centrifugation of equal amounts of native DNA and DNA denaturated by heating *Curve B* Denaturated DNA and native DNA added to suspensions of meningococci (Ml 8 *his arg cp*+) before cell lysis and extraction of nucleic acids (—— = Absorbance – – = Radioactivity)

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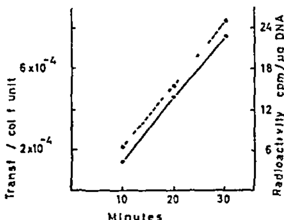


Fig 4 Transformation efficiencies and specific labelling of the DNA peak in density gradients as a function of duration of exposure of the recipient cells to ³H DNA Transformation — Radioactivity)

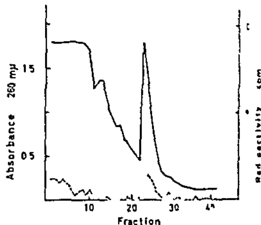


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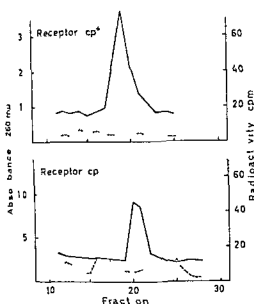


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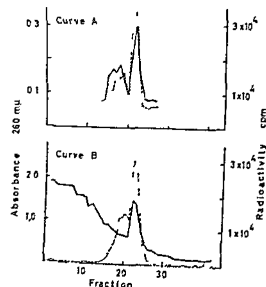


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of this material is evenly distributed as a very low background radioactivity in the CsCl density gradients This background activity seems to correspond to that found by *Lacks et al* (13) in the pneumococcus transformation Since this activity is also observed upon the use of *E coli* DNA specifically labelled in thymine it may represent donor DNA probably fragmented by the DNase used to terminate the uptake which adheres to the cells

A labelled peak corresponding to double stranded DNA is constantly found in extracts from transformed meningococci Because the specific labelling of the DNA peak increases with time of exposure to DNA and is proportional to the transformation efficiency, this uptake may correspond to the uptake specifically connected with transformation which was previously described (9)

In pneumococci *Lacks et al* suggested that the initial products of entry of DNA were equal amounts of single strands and 5 deoxyribonucleotides The latter could be isolated from cell lysates partly as further break down products as a dialyzable fraction The attempts to demonstrate radioactivity corresponding to single stranded DNA during the transformation of *N meningitidis* were altogether negative and in contrast to the situation in pneumococci very little dialyzable labelled material could be found in extracts from cells transformed with radioactive DNA

This does not necessarily mean that single strand separation does not occur (3 22) But the single strands bound to the cells or present in the interior of the cells at any time must represent a very small fraction of the total DNA If the transforming DNA is broken down during the transformation of *N meningitidis* it seems that the breakdown products are lost during the washing of the cells

The experiments with ³H labelled *E coli* DNA corroborate the previous finding that in *N meningitidis* transformation the step leading to DNase insensitivity is specific for homologous DNA (9) This is in striking contrast to the findings from *B subtilis* (3 19) and pneumococcus (13) If we adopt the hypothesis that in competent *B subtilis* cells DNA penetrates to a location between cell wall and membrane where it is not accessible for the DNase added to interrupt transformation (1, 11 15), we must also assume that this process is non specific in the sense that any double stranded DNA of proper molecular size may penetrate But the following step the transport through the membrane could well be specific for homologous DNA In fact it seems that no experiments have been done to observe whether the heterologous DNA penetrates the cell membrane of the recipient (1) It is conceivable that competent *N meningitidis* cells lack the DNase insensitive sites outside the membrane suggested for *B subtilis* In this case the irreversible uptake in *N meningitidis* (9) would directly measure the DNA that has penetrated the membrane whereas in *B sub* it measures the DNA inside the membrane plus that located at the DNase insensitive sites It may also be suggested that integration and penetration of the membrane represent the same step in *N meningitidis* transformation

An extremely small accumulation of radioactivity corresponding to double stranded DNA is also found in the genetically incompetent variant of the meningococcal Strain M1 after exposure to labelled DNA The amount is too small to be demonstrated in the technique previously used (9) The rudimentary uptake seems to be specific for homologous DNA since it cannot be reproduced with *E coli* DNA Thus it has the characteristics of the uptake *connected* with transformation in the competent variant although on a very much lower scale Whether the lack of significant transformation indicates that the DNA taken up cannot result in a true transformation or whether the number of transformants obtained is too small to raise the number of transformants significantly above that of the background mutation is not known

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ANTIBODY FORMATION IN CELL CULTURES

2 *The Effect of Rabbit Peritoneal Exudate Cells on Secondary in vitro IgG and IgM Antibody Responses to Poliovirus*

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The role of rabbit peritoneal exudate cells (PEC) in the induction of secondary *in vitro* antibody responses to poliovirus was studied IgG antibody responses were inhibited by the addition of syngeneic or allogeneic PEC to sensitized lymphoid cells of spleen origin prior to restimulation with viral antigen Stimulation with macrophage associated antigen after *in vitro* uptake by PEC, caused a similar inhibition In contrast, antigenic restimulation of lymphoid cells 30 minutes prior to the addition of PEC induced secondary IgG antibody responses *In vivo* stimulation with PEC associated poliovirus also appeared less efficient than free virus in inducing IgG antibody formation PEC had no clearly demonstrable effect on secondary *in vitro* IgM antibody responses Poliovirus was rapidly taken up by PEC, the uptake reaching a maximum within about 40 minutes An initial decline in the infectivity of PEC-associated virus was noted after 5 to 10 minutes interaction and more than 85 per cent of this virus was rendered noninfectious within 30 minutes PEC inhibited blastogenesis in antigen restimulated spleen cell cultures

Macrophages are known to be involved in the uptake of particulate material, foreign proteins and cells In recent years they have also been implicated as having a role in the immune response to certain antigens Some of these data are, however, conflicting Thus antigen-containing macrophages or RNA-extracts from such cells have been reported (*Fishman and Adler* 1963, *Fishman et al* 1965, *Askonas and Rhodes* 1965, *Feldman and Gallily* 1967, *Argyris and Askonas* 1968 *Unanue and Askonas* 1968a, *Mitchison* 1969 *Hoffman* 1970) to be capable of priming lymphoid cells for an immune response while other authors (*[illegible]* 1948, *Perkins and Makinodan* 1965 *[illegible]* 1966, *Parkhouse and Dutton* 1966) concluded that the macrophages competed with the lymphoid cells in the immune response Furthermore the nature of the interaction between macrophages and lymphoid cells is not clear as yet It is not known whether the macrophages 1) act by presenting the antigen to the lymphocyte surface in a manner which permits efficient interaction with specific antibody-like receptors 2) have a trophic or regulatory effect on division and/or differentiation of lymphoid cells [illegible] modify certain antigens (antigen processing) leading to an enhancement of their immunogenicity or 4) compete with lymphoid cells [illegible] rapidly catabolize

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We have earlier described (*Svehag* 1964 1965, *Svehag et al* 1968) a system for long term cultivation of lymphoid cells in which quantitative and qualitative aspects of secondary antibody responses to poliovirus were studied The principal aim of the present investigation was to study the possible role of peritoneal exudate cells (PEC) in the induction of secondary antibody responses in this *in vitro* system

MATERIALS AND METHODS

Antigen The Brunhilde strain, type 1, of poliovirus (PV) was grown on HeLa cell monolayers ^3H poliovirus was prepared in the same cells by prelabelling for 20 hours (3 μc ^3H leucine/ml leucine deficient Eagle's basal medium), infecting with a virus multiplicity of 10 and postlabelling with 6 μc/ml Eagle's medium containing 5 per cent dialysed calf serum Virus stocks were dialysed against phosphate (0 01 M) buffered physiological saline pH 7 3 (PBS) and partly purified by banding in CsCl solution (average density 1 34) prior to storage at —20°C The infectivity of virus stocks was assayed on HeLa monolayers and expressed in plaque forming units (PFU) per ml of virus suspension

Immunization procedure Four months old rabbits were immunized intravenously with 10^9 10^9 PFU poliovirus at various times prior to sacrifice

Spleen cell cultures Rabbits were splenectomized aseptically under nembutal anaesthesia one to five weeks after immunization with poliovirus The spleens were placed in ice cold medium, Eagle's basal medium or Parker 199 supplemented with with 15 per cent foetal calf serum and antibiotics The organs were cut into small fragments, washed in medium and dispersed in a Borrel sieve The separated cells were filtered through gauze into a centrifuge bottle containing 6 per cent Dextran 150 and 0 45 per cent EDTA in PBS and the cell suspension was centrifuged in the cold for 10 minutes at *about 3000 G* The top fraction of the packed cells containing lymphoid cells and granulocytes *was collected* the cells counted, and 1 to 2×10^7 cells were seeded on Millipore membrane filters (0 45 *mμ*) which had previously been glued to the top of lucite cylinders Each cylinder was placed in a depression *of a plastic tray* 0 5 ml medium was added and the cells were covered with a piece of porous paper which reached down into the medium The tray was finally covered with a plastic film and placed in a 37°C incubator in an atmosphere of 6 per cent CO_2 in humidified air The culture medium was replaced every second or third day and titrated for PV neutralizing antibody by the plaque inhibition technique on HeLa monolayers The specificity of the *in vitro* response was established by assaying the media against serologically unrelated viral antigens

Peritoneal exudate cells (PEC) Rabbits were injected intraperitoneally with 30–40 ml 0 1 per cent peptone broth and 3 days later the peritoneal cavity was washed out with PBS containing 0 25 per cent EDTA and 5 u heparin/ml The peritoneal exudate contained about 80 per cent macrophages and the remaining cell population was made up of mesothelial cells, granulocytes and small lymphocytes

Magnetic technique for the removal of phagocytic cells from lymphoid cell preparations 200 mg of ferrum reductum (Merck AG), suspended in physiological saline, was administered slowly intravenously to rabbits and the animals were splenectomized under nembutal anaesthesia 15 minutes after the injection A spleen cell suspension was prepared and fractionated in 6 per cent Dextran 150 by centrifugation The top fraction of the packed cells, containing most of the lymphoid cells and a minor part of iron containing cells, was serially transferred into petri dishes mounted over a magnet The phagocytes were held to the bottom of the dish by the magnetism while the other cells were transferred by gently flushing the dish with medium

Staining of cells from membrane cultures A sample of cultured cells was withdrawn from the membrane with the aid of a Pasteur pipette, suspended in Parker 199 and sedimented onto a glass slide in a specifically designed centrifuge rotor The slide was fixed and stained with methyl green pyronine (T G Gurr, London, England) or May Grunwald Giemsa

Antibody titration Sera and cell culture media were titrated for poliovirus neutralizing antibody by mixing equal volumes of virus (100 PFU) and varying dilutions of serum or medium The mixtures were kept for 4 hours at room temperature plus overnight at 4°C and were assayed without dilution on HeLa monolayers *for virus survivors* The antibody activity was expressed in per cent virus neutralization or in 50 per cent neutralizing units defined as the serum dilution at which the plaque formation was reduced by half

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Acta path microbiol scand Section B 79 572 580, 1971

ANTIBODY FORMATION IN CELL CULTURES

2 *The Effect of Rabbit Peritoneal Exudate Cells on Secondary in vitro IgG and IgM Antibody Responses to Poliovirus*

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gradient and the gradient centrifuged in a Spinco model L centrifuge using a SW 39 rotor at a maximum centrifugal force of 135 000 G for 22 hours. Fractions (0.2 to 0.3 ml) were collected dropwise through a hole in the bottom of the tube. Protein determinations and antibody titrations were performed on each fraction.

Reduction and alkylation of antibodies. Tissue culture media were incubated with equal volumes of PBS containing 0.4 M 2-mercaptoethanol at room temperature for 3 hours and subsequently alkylated for 40 minutes at 4°C with 0.04 M iodoacetic acid in 0.01 M Tris-HCl buffer, pH 8.0. Control samples were incubated with PBS or iodoacetic acid only. To remove excess reagents, all samples were dialysed in the cold against PBS.

Immunoelectrophoresis. The microtechnique of *Scheidegger* (1955) was used.

Protein determination. Protein concentrations were measured by absorbance at 280 mμ in a Beckman DU spectrophotometer in a 1.0 cm wide quartz cuvette.

Ultraviolet light inactivation of virus. Poliovirus was inactivated by ultraviolet light (UV) in a constantly agitated Petri dish placed 20 cm from a 15 watt Sylvania germicidal lamp. This treatment resulted in a reduction of six $\log_{10}$ of virus infectivity in 4 minutes.

In vitro stimulation with antigen. Fractionated spleen cell suspensions were mixed with various antigen multiplicities (physical virus particles/cell) of CsCl-banded and UV-irradiated PV. The cells were incubated for 30 minutes at 37°C, washed twice, counted and seeded on membrane filters. Controls included spleen cells incubated with measles virus or PBS.

RESULTS

Effect of Allogeneic or Syngeneic PEC on the Induction of Secondary in vitro Antibody Responses

It was investigated whether the addition of allogeneic PEC to the lymphoid cells prior to stimulation with antigen had a potentiating effect on the *in vitro* antibody response. In these experiments rabbits which had been sensitized twice with poliovirus *in vivo* were splenectomized, the spleen cells were fractionated in Dextran 150 and the top fraction was divided into several aliquots. When such aliquots of lymphoid cells were mixed with normal allogeneic PEC prior to their stimulation with a sufficiently high antigen multiplicity to induce secondary IgG

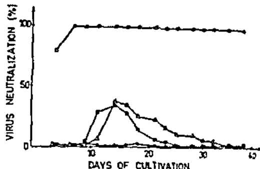


Fig. 1. The effect of allogeneic PEC on the secondary *in vitro* antibody response to poliovirus. The rabbit received two intravenous injections with poliovirus (10^9 PFU), 16 and 7 days prior to splenectomy. The spleen lymphocytes were divided into three aliquots, two of which (□ Δ) were restimulated with an antigen multiplicity (virus particles/cell) of 50. To one of these aliquots (2×10^7 cells) was added 5×10^6 PEC prior to antigenic stimulation (Δ). The 3rd aliquot (■) was not restimulated. Culture medium only (●). The media were tested at a dilution of 1:4.

antibody responses in the control cultures, these responses were wholly or partially inhibited (Fig. 1). The ratio between lymphocytes and PEC was varied from 8 to 2 and the degree of inhibition was greater when the lower ratios were used. A weak antibody response, the kinetics and mercaptoethanol sensitivity of which indicated that it contained IgM antibodies, was however observed. Note that a transient "spontaneous" IgM antibody response occasionally was observed 8 to 12 days after cultivation of the sensitized lymphoid cells in the absence of deliberate restimulation with PV (Fig. 1).

The rather marked inhibition of the *in vitro* antibody response was unexpected. It was necessary to compare these results with those obtained in the syngeneic cell combination. In these experiments sensitized lymphoid cells of spleen origin and PEC were obtained from the same animal. Although the inhibition of the secondary IgG antibody responses was more modest in these experiments, the addition of syngeneic immune macrophages did not in any case result in a potentiation of the *in vitro* antibody response.

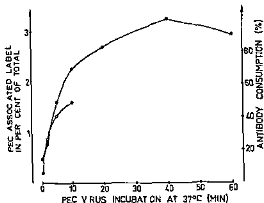


Fig 2 The uptake of ³H poliovirus by PEC (1.5×10^7 cells) *in vitro* at 37°C Aliquots of the cells were removed after varying time intervals washed three times and their radioactivity (●) was determined The capacity of washed freeze thawed cell samples to absorb antibodies to polio virus (○) was also determined for the initial 10 minutes period of virus cell interaction

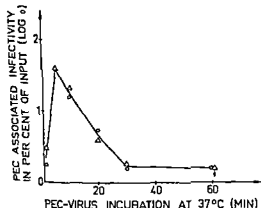


Fig 3 Infective centre titrations of PEC incubated with poliovirus at 37°C 2×10^6 macrophages were reacted with a virus multiplicity of 50 and aliquots of the cells were washed three times and assayed for infectious cell associated virus after 1 5 10 20 30 and 60 minutes on HeLa cell monolayers The curve represents the results of two experiments

Uptake of Poliovirus by PEC

The rate of poliovirus uptake by rabbit PEC was studied by tracing the label and infectivity of ³H poliovirus and the antigenicity of PEC associated virus was determined in antibody consumption tests using freeze thawed cell samples Maximal uptake of the label was reached within about 40 minutes at 37°C (Fig 2) This however, constituted only 3 0 to 3 5 per cent of the total amount of antigen added A rapid decline of PEC associated virus infectivity was observed already after 5 to 10 minutes of poliovirus PEC interaction (Fig 3) At this time a beginning deviation between the curves representing uptake of labelled virus and antigenicity of PEC associated virus was also suggested (Fig 2) More than 85 per cent of the macrophage associated poliovirus had lost its infectivity within 30 minutes indicating a rapid breakdown of the virions

In vitro Stimulation with Macrophage Digested Poliovirus

In these experiments poliovirus was allowed to react with rabbit PEC for 30 minutes at 37°C before adding the washed cells to the lymphocyte cultures In the majority of cases this resulted in a clear inhibition of the secondary *in vitro* IgG antibody responses (Fig 4) while weak transient IgM responses were observed Such transient mercaptoethanol sensitive 19S antibody responses were

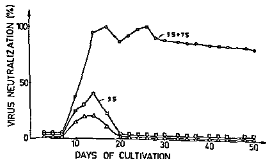


Fig 4 The effect of allogeneic PEC on the secondary *in vitro* antibody response to poliovirus The rabbit received one intravenous injection of polio virus (5×10^9 PFU) 35 days prior to surgery The spleen lymphocytes were divided into three aliquots each containing 2×10^7 cells two of which were stimulated with an antigen multiplicity of 2 One aliquot (○) received the antigen 30 minutes prior to adding the PEC (2×10^6 cells), the other (□) received macrophage reacted (30 minutes) antigen The 3rd aliquot (△) was not restimulated The media were tested at a dilution of 1 4

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Acta path microbiol scand Section B 79, 581–587, 1971

THE EFFECT OF PREGNANCY ON REJECTION OF ALLOTRANSPLANTED HEARTS IN THE RABBIT AND RAT

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The effect of pregnancy on the rejection time and pattern of heterotopic heart allografts has been studied in outbred rabbits and in inbred rats Female rabbits pregnant by donor unrelated males rejected within normal periods of time In the rat system recipients were transplanted during 1) intrastrain pregnancy 2) interstrain non donorrelated pregnancy and 3) during interstrain donor specific pregnancy No significant prolongation of allograft survival time was found regardless of the genetic constitution of the foetuses It is concluded that pregnancy induced immunosuppression cannot explain the success of the foetal allograft to survive the period of gestation

An allograft is a transplant between members of the same species which normally elicits an immune reaction in the unmodified host leading to the destruction of the grafted tissue

This reaction depends upon the immunogenetic disparities between donors and hosts

The foetus contains some of the genetic characteristics of the father and may in normal pregnancies be looked upon as an allograft to the mother This natural allograft however seems to escape immunological destruction during the period of gestation and is an exception to the general rule of allotransplant rejection While the mechanisms which may account for this successful transplant are not clear, it has been suggested that one of the factors may be a decreased immunological reactivity in the mother to the antigens of her foetus

Attempts to demonstrate a state of general immunosuppression in the mother during pregnancy by subjecting her to various antigenic stimuli and studying the immune response have not been consistently successful In a few isolated instances immunosuppression has been demonstrated during pregnancy, as a delay in rejection of skin and tumor transplants arising from paternal strains was found (1, 2, 6, 13) The work of others (7, 8, 12) has not supported this assumption The present investigation was undertaken to study the effects of intra- and interstrain pregnancy in an experimental system for studies of organ allograftrejection in which an accessory heart transplant is used as allogeneic graft in rabbits and inbred rats

Received 29 1 71

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MATERIAL AND METHODS

Randomly bred virgin female rabbits were mated to an unrelated male from another breeding centre During the following 2–15 days she received an accessory cervical heart allograft from a young rabbit (1–2 months of age), bred by a third centre The technique of transplantation and the way of determination of the endpoint of rejection has been described previously (1) Rabbits that did not give birth or abort during 4–5 weeks following mating served as controls together with non mated virgin females

Rats of the inbred strains Wistar, Fischer and BN were employed The genetic homogeneity of the rat strains has been confirmed by the permanent survival of intrastrain skin and heart transplants

In the first series 3 groups of virgin female Wistar (3–6 month of age) were mated to one of the following 1) Wistar males, 2) Fischer males 3) BN males Within 1–3 weeks following mating the Wistar females in each of the groups received an accessory cervical heart transplant from a Fischer male (2 3 months of age) or from a male Wistar Fischer F 1 hybrid of the same age

In the second series virgin Fischer females served as recipients mated intra or interstrain In these cases Wistar rats served as donors for the heart transplants

Operative performance has been detailed elsewhere (4)

Recipients that did not give birth or abort following and during the experimental period served as controls together with non mated virgin females

The duration of pregnancy was calculated retrospectively from the day of parturition A gestation period of about three weeks allows separation of events occurring during early middle and late pregnancy

RESULTS

A summary of the data obtained is recorded in Tables 1–3 and Figs 1–3

In control rabbits cardiac allografts were rejected between 6 and 16 days post transplantation as judged by the electrocardiogram the pulsatile activity and the histological appearance Rabbits transplanted during interstrain pregnancy rejected their grafts from unrelated donors within the same range of time (Table 1, Fig 1) whether transplanted during early, middle or late pregnancy The parturition and the litters seemed unaffected by the procedures The clinical and histological features of rejection were identical to those in the control group (5)

Non mated virgin and sterile mated female Wistar rats rejected their Fischer allograft between 8 and 12 days post transplantation The intrastrain (Wi × Wi) and interstrain (Wi × BN and Wi × Fi) pregnant recipients rejected the Fischer cardiac allografts within periods of time that were not significantly different from those of the controls (except for a single transplant that survived for 52 days) regardless of the genetic constitution of the foetuses (Table 2, Fig 2) The histological and clinical features were identical in the various groups except for late transplants surviving delivery, which clinically showed a slow down for a few days in the rejection reaction (i e increase in tension of the transplant and decrease in voltage)

The results from the reverse donor host combination (Fischer recipients—Wistar donors) were consistently in accordance with the above

Almost all allografting experiments performed during pregnancy could be carried

TABLE 1 *Heart Transplantation to Pregnant Rabbits*

Recipient	Donor	Pregnant by	Day of pregnancy when transplanted	Rejection time days
Black Alaska	White	Grey lop eared	2	7
Brown	White	Brown and white	4	8
Brown	Grey	Grey lop eared	8	10
Brown lop eared	White	Brown and white	15	15
White	Brown	Brown and white	18	6
White	Black Alaska	Brown and white	20	13

TABLE 2 *Heart Transplantation to Pregnant Wistar Rats*

Strain of recipient	Strain of donor	Pregnant by	Day of pregnancy when transplanted	Rejection time days
W_1	F_1	W_1	2	9
W_1	F_1	W_1	3	8
W_1	F_1	W_1	8	9
W_1	F_1	W_1	10	9
W_1	F_1	BN	1	8
W_1	F_1	BN	1	9
W_1	F_1	BN	1	9
W_1	F_1	BN	3	16
W_1	F_1	BN	4	8
W_1	F_1	BN	5	9
W_1	F_1	BN	6	9
W_1	F_1	BN	6	11
W_1	F_1	BN	7	15
W_1	F_1	BN	10	8
W_1	F_1	BN	10	12
W_1	F_1	BN	11	9
W_1	F_1	BN	15	52
W_1	F_1	BN	17	11
W_1	F_1	BN	20	9
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W_1	F_1	F_1	2	8
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W_1	W_1F_1 F_1	F_1	7	7
W_1	W_1F_1 F_1	F_1	7	9
W_1	F_1	F_1	7	9
W_1	W_1F_1 F	F_1	8	7
W_1	W_1F_1 F_1	F_1	8	8
W_1	W_1F_1 F_1	F_1	10	16
W_1	W_1F_1 F_1	F_1	12	13

out without any obvious side effects in the pregnant host or for the outcome of the pregnancy except for three litters in which a small number of stillbirths occurred

Isogeneically transplanted rat hearts have survived in this laboratory for periods exceeding 400 days and serve as controls for the operative method used as well as for isogeneity of the rat strains employed

Cases with complications in the material which could easily be evaluated have been excluded from the tables

Recipients investigated for cytotoxic antibodies to donor strain lymphocytes were found to possess cytotoxins at the time of rejection* This was also found in *recipients* carrying a pregnancy with donorspecific genetic constitution

DISCUSSION

The successful take of the foetal allograft still remains one of the great mysteries in transplantation *immunology* Various hypotheses have been advanced including theories of 1) the uterus as an immunologically privileged site, 2) antigenic immaturity of the foetus 3) anatomical or physiological barriers

* Observations to be detailed in a later report

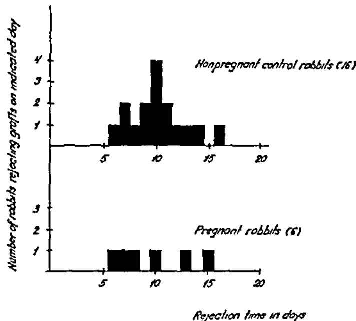


Fig 1 Rejection time of rabbit cardiac allografts by pregnant and nonpregnant rabbits Figures in parentheses represent numbers in each group

TABLE 3 *Heart Transplantation to Pregnant Fischer Rats*

Strain of recipient	Strain of donor	Pregnant by	Day of pregnancy when transplanted	Rejection time days
F1	W1	F1	1	8
F1	W1	F1	3	8
F1	W1	F1	5	8
F1	W1	F1	8	7
F1	W1	F1	15	8
F1	W1	BN	1	8
F1	W1	BN	6	7
F1	W1	BN	7	7
F1	W1	BN	7	8
F1	W1	BN	11	13
F1	W1	BN	14	8
F1	W1	W1	1	7
F1	W1	W1	3	8
F1	W1	W1	5	8
F1	W1	W1	5	8
F1	W1	W1	7	9
F1	W1	W1	14	9

between maternal and foetal vascular systems, and 4) decreased immunological reactivity of the mother during pregnancy (15) In support of the latter hypothesis *Heslop et al* (6) found that skin allograft survival in pregnant rabbits was prolonged for transplants applied on or near the 22nd day of pregnancy compared to skin transplantation performed at other times *Lanman* (7) stated from work done in his laboratory that this apparent immuno suppressive effect of pregnancy on skingraft rejection in rabbits was only slight and statistically insignificant *Halasz & Orloff* (3) who also studied skin transplants on pregnant rabbits showed that the pregnant doe produces both cellular and

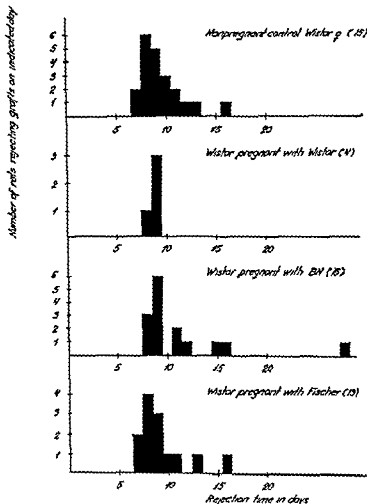


Fig 2 Rejection of allotransplanted Fischer and Wistar Fischer F_1 hybrid hearts in female Wistar recipients The total number in each group is given in parentheses

humoral antibodies They succeeded in demonstrating a slightly prolonged survival time of first set allografts even early in the course of pregnancy but found that second set grafts were rejected within normal periods of time *Woodroff* (15) working on the same subject using transplants of embryonic tissues to subcutaneous sites in pregnant rabbits and rats could demonstrate a typical immune response to the tissues of their own embryo while embryos remaining in the uterus were unaffected

Other systems have been tested to evaluate if any immunosuppression appears during pregnancy Thus *Merrit* & *Galton* (9) observed that antibody production was not significantly altered when pregnant mice hamsters and guinea pigs were challenged with the antigens Sheep red blood cells, bacterial endotoxin and bovine gammaglobulin *Medawar* & *Sparrow* (8) found no difference in survival times of skin grafts in pregnant and nonpregnant mice nor did *Simmons et al* (12) in contrast to the results of the work of *Valone* (13) He concluded using other strains of mice that gestation had a beneficial effect on skin allograft survival especially if transplanted late in the course of pregnancy In allogeneic mouse tumour systems decrease in maternal immune reactivity has been found but not in all instances (1 2)

The experimental evidence presented in the present paper substantiates the concept

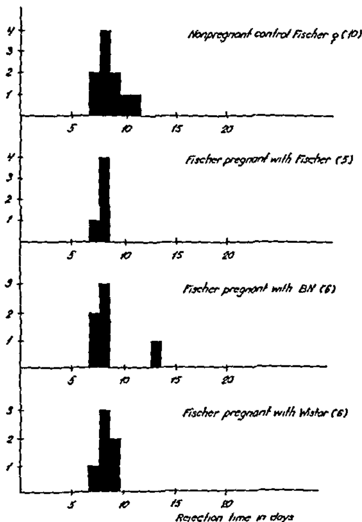


Fig 3 Rejection of Wistar hearts by female Fischer recipients Figures in parentheses represent numbers of animals in each group

that pregnancy in rabbits and rats has no effect on the immune response to allografts transplanted across major histocompatibility barriers Even transplants antigenetically identical to the foetus undergo normal rejection in the pregnant rat which also may form detectable cytotoxic antibodies against the donor (and foetal) antigens without adverse effects on the litter

Transplants made during very late pregnancy and following delivery have been excluded from this investigation because such transplants are dealt with by the recipient in the postpartum period thus constituting an entirely new situation which will be the subject of later communication

The organ allograft assay system employed in the present experimental study was chosen for several reasons One of these was the advantage of being able to make a very exact determination of rejection time in this system Another was based on the theoretical possibility of getting an allograft immune response to the endothelium lined organ transplant different from that of the commonly used skin transplant which suffer a long period of avascularity and which may release its antigens and sensitize the recipient in a different way Considerable differences in survival times of skin and organ transplants in other situations have been reported (10 11, 14) It was thought that the heart graft might pre

sent its antigens and immunize the host in a way analogous to that of the foeto placental allograft

From the experimental data reviewed and presented we conclude that pregnancy-induced mechanisms of decreased immunological activity are operational only in some experimental combinations, and as a whole cannot explain the success of the foetal allograft to survive the period of gestation

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This work was supported by grants from *Kong Christian den Tiendes Fond, Statens lægevidenskabelige Forskningsråd* in Denmark and a scholarship under the *University of Aarhus*

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Acta path microbiol scand Section B 79, 588-594 1971

LYMPHOCYTE STIMULATION BY A COMPLEX OF PHYTOHAEMAGGLUTININ AND RED CELL MEMBRANE

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Lymphocytes purified from human blood responded poorly to phytohaemagglutinin (PHA) The response was increased by addition of red cell membranes but not by soluble products from red cells PHA-coated red cell membranes stimulated purified lymphocytes The response obtained was higher than that caused by an optimal concentration of PHA in soluble form The results indicate that a complex of PHA and red cell membrane has higher lymphocyte stimulating activity than has free PHA

It has previously been found that red blood cells (RBC) play a significant role in phytohaemagglutinin (PHA)-induced lymphocyte stimulation (*Tärnvik* 1970 b) The response to PHA was low when few blood cells other than lymphocytes were present in the culture The addition of RBC together with the PHA to the purified lymphocytes resulted in a response like that of an essentially non purified lymphocyte preparation The present paper describes results which support the assumption that PHA bound to the RBC membrane has higher lymphocyte stimulating activity than has free PHA

MATERIALS AND METHODS

Purification of lymphocytes Venous blood was obtained from healthy blood donors Lymphocytes were isolated from 300 ml blood by filtration through a nylon fibre column followed by differential centrifugation in colloidal silica polyvinyl pyrrolidone (*Tarnvik* 1970 a)

RBC membranes were counted in a Burker cytometer

Culture medium The culture medium consisted of 80 per cent Parker TCM 199 (Flow Laboratories Irvine Ayrshire Scotland) and 20 per cent pooled inactivated (56°C 30 min) human serum The medium also contained 150 units of benzyl penicillin (KABI AB Stockholm Sweden) and 150 µg of streptomycin sulphate (Glaxo Laboratories Ltd Greenford England)

Phytohaemagglutinin PHA was purified from *Phaseolus vulgaris* (Weibull no 44 from Weibullsholm Plant Breeding Institute Landskrona Sweden) by the technique described by *Borjeson et al* (1964), but with omission of the chromatography step A single batch of PHA stored in aliquots at —20°C was used throughout the investigation

Preparation of RBC RBC were obtained from the same blood sample as used for lymphocyte preparation After filtration through a nylon fibre column the blood was centrifuged and the plasma was discarded The filtered RBC were suspended to appropriate densities in culture medium

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Preparation of membranes and soluble products of RBC RBC membranes were prepared by hypotonic treatment Blood from the same sample as used for the lymphocyte preparation was filtered through a nylon fibre column It was then centrifuged in 1 5 ml aliquots After removal of the plasma, 7 5 ml of TCM 199 were added This suspension was distributed in 1 5 ml aliquots in centrifuge tubes and 6 ml of deionized water were added to each tube RBC membranes were collected by centrifugation at 2,800 rpm (1,600 g) for 45 min The supernatant fluid was recentrifuged at 30,000 rpm (63,000 g) for 30 min to remove remaining insoluble products The resulting supernatant fluid was used as "soluble products from RBC"

The button of RBC membranes was transferred to another centrifuge tube Great care was taken to avoid transfer of still unlysed RBC in the very bottom of the tube The membranes were washed twice in physiological saline and suspended in culture medium

PHA coating of RBC membranes and RBC RBC membranes and RBC were suspended in culture medium to give 5×10^8 cells or membranes per ml of suspension PHA (1 2 mg) was dissolved in 2 ml of physiological saline and added under gentle stirring to 10 ml of the suspension Stirring was continued for 2 hours at 37°C and 40 ml of saline were added at the end of that period The PHA treated RBC membranes and RBC were washed twice in 10 ml of saline For control purposes RBC membranes and RBC not treated with PHA were used These membranes and cells were suspended to the appropriate densities in culture medium Due to agglutination, PHA treated cells or membranes could not be counted They were, however, suspended in the same way as the untreated ones

Measurement of lymphocyte stimulation The lymphocytes were suspended in culture medium to $0 7 \times 10^6$ cells per ml If not otherwise stated, 30 µg of PHA in 50 µl of deionized water were added per ml of lymphocyte suspension The suspension was distributed in 1 5 ml aliquots in 16×110 mm round bottomed glass tubes loosely capped with aluminum foil The cultures were incubated for 62 hours at 37°C in humidified air supplemented with 5 per cent (v/v) CO_2 Fourteen hours before the end of the culture period, 1 5 µCi of [methyl ^{3}H] thymidine (specific activity 6 7 Ci per mmole New England Nuclear Corp, Boston, Massachusetts) in 30 µl of deionized water was added to each tube The radioactivity of the ^{3}H thymidine incorporated into DNA was measured (*Borjeson et al* 1966, *Tarnvik* 1970 b)

Soluble and insoluble RBC constituents were added to the lymphocyte cultures in order to test their effect on PHA induced lymphocyte stimulation These products were dissolved or suspended in culture medium to the appropriate concentrations, and 0 2 ml was added to each lymphocyte culture so that the final volume was 1 7 ml

RESULTS

Effect of RBC, RBC membranes, and soluble products of RBC on the lymphocyte response to PHA Lymphocytes were incubated in the presence of PHA The effects of RBC, RBC membranes, and soluble products of RBC on the incorporation of ^{3}H-thymidine into DNA were tested Incorporation was higher in the presence than in the absence of RBC, which is in accordance with previous results (*Tärnvik* 1970 b) The enhancement of incorporation observed in the presence of RBC could be obtained by substituting RBC membranes but not by substituting soluble products of RBC (Table 1) Thus, in the presence of 10^7 RBC membranes per ml of culture, *i e*, at an RBC membrane lymphocyte ratio of 14, the incorporation of ^{3}H-thymidine was higher than in the absence of RBC membranes At an RBC membrane density of 10^6 membranes per ml, the effect of the membranes was less evident

The lymphocyte response to PHA-coated RBC and PHA coated RBC membranes The lymphocyte stimulating activities of PHA-coated RBC and PHA-coated RBC membranes were investigated When lymphocytes were incubated alone the incorporation of ^{3}H thymidine into DNA was low (Table 2) The presence of RBC or RBC membranes did not increase the incorporation However, PHA-coated RBC induced a high incorporation in the lymphocytes, even in the absence of PHA in soluble form PHA-coated RBC membranes apparently induced still higher incorporation

The incorporation induced by PHA coated RBC membranes was, in fact, higher than that induced by PHA in solution, provided that no RBC or RBC membranes were present together with the PHA solution (Table 2)

The washing of the RBC membranes after PHA-treatment was judged to be effective, since the supernatant fluid collected after the

TABLE 1 *Effect of RBC Membranes, Soluble Products of RBC and Intact RBC on the Lymphocyte response to PHA*

Dilution of added preparation	*Preparation added* RBC membranes	Soluble products from RBC	Intact RBC	Culture medium
Exp no 1				
1/1	48 0±6 6*	8 5±1 5	52 6±1 4	11 3±2 0
1/10	17 4±7 0	11 1±2 0	30 0±3 0	
Exp no 2				
1/1	35 0±7 0	11 9±2 6	38 6±4 2	13 2±2 0
1/10	20 9±5 0	14 9±4 6	19 6±5 5	

*Mean counts × 10³ per min ± S.D of 5 cultures

Purified lymphocytes were incubated in the presence of PHA RBC membranes or intact RBC were suspended to a final density of 10^7 (1/1) or 10^6 membranes or cells per ml Soluble products obtained from the corresponding numbers of RBC were added to other cultures To control cultures culture medium alone was added The incorporation of ^3H thymidine into DNA was measured

TABLE 2 *The Lymphocyte Response to PHA coated RBC Membranes and PHA coated RBC*

Material added	Counts per min Means ± S D of 5 cultures — Exp no 1 — Addition of PHA to culture medium: absent	present	Exp no 2 — Addition of PHA to culture medium: absent	present
PHA coated RBC	2,408±573		5 614±3 110	
Untreated RBC	71±10	11,604±3 235	78±27	30 373±10 696
PHA coated RBC membranes	6,776±2 680		15 109±6 962	
Supernatant from the last washing of PHA coated RBC membranes	67±26	930±348	128±201	3 849±582
Untreated RBC membranes	69±14	6 489±1 431	79±59	26 554±6 759
Liquid from tube containing PHA solution handled in the same way as tubes with PHA coated RBC membranes	60±7		69±24	
Culture medium	51±15	983±385	260±302	4 079±854

Purified lymphocytes were incubated in the presence or absence of PHA PHA coated or untreated RBC membranes or RBC were added to the cultures to a final density of 10^8 cells or membranes per ml of culture The incorporation of ^3H thymidine into DNA was measured

last washing step did not cause increased incorporation of ^3H-thymidine into DNA (Table 2) To exclude the possibility that PHA had formed a complex free from the RBC membranes but not removable by the washing procedure a PHA solution was treated in the same way as in the treatment of membranes, and the final solution was added to lymphocyte cultures This solution did not cause increased incorporation of ^3H thymidine into DNA (Table 2)

The possible release into the culture

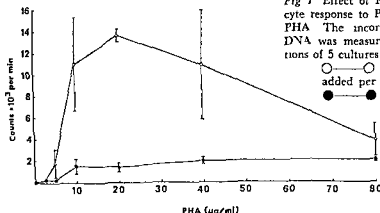


Fig 1 Effect of RBC membranes on the lympho cyte response to PHA at various concentrations of PHA The incorporation of ^{3}H thymidine into DNA was measured Means and standard devia tions of 5 cultures are indicated
○——○ 10^9 autologous RBC membranes added per ml of culture
●——● no RBC membranes added

TABLE 3 *The Lymphocyte Response to Soluble Products Released from PHA Coated RBC Mem branes*

Products added	Counts per min ± S D of 5 cultures
PHA-coated RBC membranes	1 106±356
Untreated RBC membranes	19±6
Supernatant of PHA coated RBC membranes	22±4
PHA and untreated RBC membranes	7 147±406

Lymphocytes were incubated for 3 days in the absence of PHA Separate tubes containing $1\,2\times10^9$ PHA coated RBC membranes in 4 ml of culture medium were incubated parallel with the lymphocyte cultures On the third day the sus pensions of RBC membranes were centrifuged at 2 800 rpm (1 600 g) for 30 min The supernatant fluid was collected and 0 2 ml of the fluid was added per lymphocyte culture to a final volume of 1 7 ml The RBC membranes were suspended in culture medium and 0 2 ml of the suspension was added to each of other lymphocyte cultures to make a final RBC membrane density of 4×10^7 mem branes per ml of culture To control cultures were added untreated RBC membranes or PHA plus untreated RBC membranes The lymphocytes were incubated for another 3 day period The incorpora tion of ^{3}H thymidine into DNA during the last 14 hours was measured

medium of soluble lymphocyte stimulating products from PHA-coated RBC membranes was tested Lymphocytes, PHA coated RBC membranes and untreated RBC membranes were incubated separately for 3 days The membranes were then separated from the incubation medium and added to the lympho-cyte cultures, which were incubated for an other 3-day period, with ^{3}H-thymidine present during the last 14 hours (Table 3) In control cultures to which untreated membranes were added the incorporation of ^{3}H thymidine was low When the PHA coated membranes were added, considerable incorporation occurred Addition of the incubation medium separated from the PHA-coated membranes did not affect the incorporation of ^{3}H thymidine Thus, lymphocyte stimulating products were not found in the solution in which PHA coated RBC membranes had been incubated

Quantitative relations between concentra tion of PHA and density of RBC membranes in lymphocyte stimulation Keeping the lym phocyte density constant at $0\,7\times10^6$ cells per ml of culture, the incorporation of ^{3}H thymi dine into DNA was measured at varying con centrations of PHA and densities of RBC membranes

Lymphocytes were incubated with 2 5 to 80 µg of PHA per ml of culture When 10^9 RBC membranes were present per ml of culture an optimal incorporation of ^{3}H thymidine into DNA occurred at 10 to 40 µg of PHA per ml and there was a decrease at 80 µg per ml of culture (Fig 1) When lymphocytes were incubated without addition of RBC membranes, the response was lower in the concentration range tested Optimal

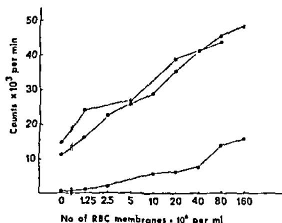


Fig 2 Effect of varying numbers of RBC membranes on the lymphocyte response to PHA The concentration of PHA was 30 μg per ml of culture The incorporation of ^{3}H thymidine into DNA was measured Three experiments with lymphocytes from different individuals are shown RBC membranes were obtained from the same blood samples as the lymphocytes The means of 3 to 5 cultures are indicated

incorporation occurred at 10 μg PHA per ml, but then the curve remained at a plateau Lymphocytes were incubated in the presence of 0 to 160$\times$10^6 RBC membranes per ml of culture, keeping the PHA concentration constant at 30 μg per ml The incorporation of ^{3}H-thymidine into DNA increased with *increasing density* of RBC membranes (Fig 2)

The effect of various densities of RBC membranes on the incorporation of ^{3}H thymidine was tested at three different PHA concentrations When the PHA concentration was 4 or 15 μg per ml of culture, the curve showed an optimum at 25 to 100$\times$10^6 membranes per ml and a decline at 100 to 200$\times$10^6 membranes per ml When the PHA concentration was *50 μg per ml the curve* did not decline even at 200$\times$10^6 membranes per ml (Fig 3)

a

Counts x10^3 per min

No of RBC membranes x 10^6 added per ml

b

Counts x 10^3 per min

No of RBC membranes x 10^6 added per ml

Fig 3 Effect of varying numbers of RBC membranes on the lymphocyte response to PHA at various concentrations of PHA in two experiments The incorporation of ^{3}H thymidine into DNA was measured The means and standard deviations of 4 or 5 cultures are indicated The concentrations of PHA were

a ○——○ 50 μg per ml ●——● 4 μg per ml

b ○——○ 15 μg per ml ●——● 4 μg per ml

DISCUSSION

Erythroagglutinating PHA adsorbs to RBC (*Barkhan & Ballas* 1963, *Kornfeld & Kornfeld* 1970) The present results indicate that a complex of PHA and RBC membrane induces lymphocyte stimulation more effectively than does PHA free in solution This suggests that enhancement of the PHA response by RBC is related to the binding of PHA to the RBC membrane Similar results have briefly been reported (*Johnson & Kirkpatrick* 1970).

There are alternative mechanisms for explaining such a role of the RBC membrane First, the spatial arrangement of PHA molecules on the RBC surface may be crucial for the triggering of lymphocyte stimulation Two or more PHA molecules arranged in a critical way may be needed to induce stimulation, for example by increasing the avidity between PHA and the PHA receptor of the lymphocyte A spatial arrangement of PHA on the RBC surface is suggested by the presence of specific PHA receptor sites on the RBC membranes (*Kornfeld & Kornfeld* 1970) Secondly, the conformation of the PHA molecule may be changed by adsorption to the RBC membrane A conformational change may lead to the exposition of a stimulating part of the PHA molecule Thirdly, the RBC membrane may be actively involved in the trigger mechanism in co operation with membrane bound PHA

The PHA dose response curve obtained in the presence of RBC membranes had essentially the same appearence as when RBC were present in the culture (*Tarnvik* 1970 b) The curve (Fig 1) showed a rapid increase at low doses an optimum, and a decrease at the highest PHA dose tested In the absence of RBC membranes the response was lower over the entire dose range tested, and no decrease was found at the highest dose The lymphocyte response to PHA increased with increasing numbers of RBC membranes in the culture (Fig 2) and reached an optimum which was dependent on the concentration of PHA (Fig 3) The magnitude of the lymphocyte response depends not only on the amount of PHA and RBC membranes, but also on the number of lymphocytes present in the cultures (*Tarnvik* 1970 b)

The results indicate that a complex of PHA and RBC membranes induces lymphocyte stimulation more effectively than does PHA added in solution, and that there are quantitative relations between PHA concentration, RBC membrane density, and lymphocyte density This suggests that PHA bound to the RBC membrane is a more effective stimulant than is free PHA, and that the bound and free forms of PHA compete for the PHA receptor sites on the lymphocytes Thus, when the PHA concentration is increased in a culture in which the densities of lymphocytes and RBC are constant (Fig 1), RBC membranes will be more and more filled with PHA This will favour the chance of contact between membrane bound PHA and lymphocytes, and the response will increase However, as the concentration of PHA increases, the amount of free PHA will also increase The response is optimal when the point has been reached at which further increase in the concentration of PHA results in exposure of the lymphocytes to proportionally more free than membrane-bound PHA As the concentration of PHA is further increased, the proportion of membrane-bound PHA reacting with the lymphocytes will further decrease, at high concentrations of PHA the lymphocyte response will be the same as in a culture containing no cells other than lymphocytes

If this interpretation is correct, the decreased response at a high concentration of PHA might be analogous to the induction of specific tolerance by high antigen concentrations (*Dresser & Mitchison* 1968, *Diener & Armstrong* 1969) If so, the presence of PHA on the RBC surface could be analogous to the presence of antigen on the macrophage surface (*Unanue & Cerrotini* 1970)

The possibility of a directly toxic effect of PHA to explain the decreased response at 80 µg PHA per ml of culture is contradicted by the present results, as in that case, a decreased

response at 80 μg per ml should be expected also in the absence of RBC membranes

Moorhead et al (1967), working with essentially nonpurified lymphocytes, have reported that the lymphocyte response to PHA increases with increasing cell proximity in the culture They suggested that the mechanism behind the increased response might be that closer approximation of the cells gives greater opportunity for cell interaction, which in turn results in activation of otherwise nonreactive lymphocytes The present results suggest that proximity between lymphocytes on the one hand and RBC carrying PHA on their surface on the other, was at least part, the basis of the phenomenon observed by *Moorhead et al* (1967)

Forsdyke (1967) has demonstrated a relationship between the PHA dose necessary to obtain optimal lymphocyte response and the serum concentration of the culture medium The present results together with those of *Forsdyke* (1967) thus indicate that there are quantitative relations between PHA concentration, lymphocyte density, RBC density, and serum concentration which, when optimal, give optimal stimulation

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Grants for this investigation have been received from *the Faculty of Medicine, University of Umeå, and from the Helge and Manhild Johansson foundation* I am grateful to Dr *Bertil Cedergren*, who kindly provided blood samples

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Acta path microbiol scand Section B 79, 595 598, 1971

CYTOTOXIC ANTIBODIES IN SERUM OF PREGNANT WOMEN AT DELIVERY

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A group of 239 pregnant women with cytotoxic antibodies in serum was compared with a group of 878 women without cytotoxins in serum at delivery according to the parameters Abortion in the history premature delivery malformation of the foetus, stillbirth pre eclampsia oedema during pregnancy arterial hypertension and albuminuria Cytotoxic antibodies were found to be of no significance according to the parameters above

The first reports on leucocyte iso antibodies formed during pregnancy were published in 1958 (14, 17) *Van Rood* (17) reported a case of a transfusion reaction in a patient who had recently been delivered of pre mature twins The patient had never been transfused before but had had five previous pregnancies Independently, *Payne* & *Rolfs* (14) showed that leuco agglutinins could arise through immunization during pregnancy

In 1964 *Terasaki* & *McClelland* (21) introduced their micro lymphocytotoxic assay and found that pregnancy could provoke the formation of maternal cytotoxins to lymphocytes *Engelfriet* (3 12) confirmed the observations made by Terasaki & McClelland Leuco agglutinins and cytotoxins formed during pregnancy were nearly always found to be directed against antigens belonging to the HL A system

The finding of leucocyte iso antibodies formed during pregnancy naturally posed the question Are leucocyte iso antibodies of clinical significance for the pregnant women, for the course of the pregnancy, or for the infant

The purpose of this investigation was to elucidate the clinical significance of cytotoxic antibodies found at delivery

MATERIALS AND METHODS

Sera from 1 994 pregnant women were investigated for the presence of cytotoxins The sera of 993 women were tested against a lymphocyte test panel consisting of 10 test donors with known HL A specificities covering the common antigens (HL A 1 2 3 9 10 11 Li Ba* 5 7 8 12 13 BB FJH R* SL LND Go-ET) and 116 gave positive reactions The 877 women whose sera gave negative reactions were excluded from the material as the possibility of cytotoxic antibodies directed against rather infrequent or yet unknown antigens could not be dismissed

1 001 women s sera were tested against lymphocytes from their husbands 123 sera were found to

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Received 23 ii 1971

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response at 80 μg *per ml should be expected* also in the absence of RBC membranes

Moorhead et al (1967), working with essentially nonpurified lymphocytes, have reported that the lymphocyte response to PHA increases with increasing cell proximity in the culture They suggested that the mechanism behind the increased response might be that closer approximation of the cells gives greater opportunity for cell interaction, which in turn results in activation of otherwise nonreactive lymphocytes The present results suggest that proximity between lymphocytes on the one hand and RBC carrying PHA on their surface on the other, was at least part, the basis of the phenomenon observed by *Moorhead et al* (1967)

Forsdyke (1967) has demonstrated a relationship between the PHA dose necessary to obtain optimal lymphocyte response and the serum concentration of the culture medium The present results together with those of *Forsdyke* (1967) thus indicate that there are quantitative relations between PHA concentration, lymphocyte density, RBC density, and serum concentration which, when optimal, give optimal stimulation

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Grants for this investigation have been received from *the Faculty of Medicine, University of Umeå and from the Helge and Manhild Johansson foundation* I am grateful to Dr *Bertil Cedergren* who kindly provided blood samples

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Acta path microbiol scand Section B 79, 595–598, 1971

CYTOTOXIC ANTIBODIES IN SERUM OF PREGNANT WOMEN AT DELIVERY

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A group of 239 pregnant women with cytotoxic antibodies in serum was compared with a group of 878 women without cytotoxins in serum at delivery according to the parameters *Abortion in the history, premature delivery, malformation of the foetus, stillbirth, pre eclampsia*, oedema during pregnancy, arterial hypertension and albuminuria Cytotoxic antibodies were found to be of no significance according to the parameters above

The first reports on leucocyte iso antibodies formed during pregnancy were published in 1958 (14, 17) *Van Rood* (17) reported a case of a transfusion reaction in a patient who had recently been delivered of premature twins The patient had never been transfused before, but had had five previous pregnancies Independently, *Payne* & *Rolfs* (14) showed that leuco agglutinins could arise through immunization during pregnancy

In 1964 *Terasaki* & *McClelland* (21) introduced their micro lymphocytotoxic assay and found that pregnancy could provoke the formation of maternal cytotoxins to lymphocytes *Engelfriet* (3 12) confirmed the observations made by Terasaki & McClelland Leuco agglutinins and cytotoxins formed during pregnancy were nearly always found to be directed against antigens belonging to the HL-A system

The finding of leucocyte iso-antibodies formed during pregnancy naturally posed the question Are leucocyte iso antibodies of clinical significance for the pregnant women, for the course of the pregnancy, or for the infant

The purpose of this investigation was to elucidate the clinical significance of cytotoxic antibodies found at delivery

MATERIALS AND METHODS

Sera from 1 094 pregnant women were investigated for the presence of cytotoxins The sera of 993 women were tested against a lymphocyte test panel consisting of 10 test donors with known HL A specificities, covering the common antigens (HL A 1 2 3 9 10 11 Li Ba*, 5 7 8 12 13 BB FJH R* SL LND Go E1) and 116 gave positive reactions The 877 women, whose sera gave negative reactions were excluded from the material as the possibility of cytotoxic antibodies directed against rather infrequent or yet unknown antigens could not be dismissed

1,001 women s sera were tested against lymphocytes from their husbands 123 sera were found o

Received 23 ii 1971

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contain cytotoxins These 123 women constituted together with the 116 women with positive reactions in the panel test the total group of 239 women with cytotoxins in serum at delivery The 878 women whose sera gave negative reactions with their husbands lymphocytes constituted the control group of women without cytotoxins in serum

The material was unselected but included no women with erythrocyte iso antibodies because *these women were not delivered at this hospital* Women suffering from acute or chronic diseases during pregnancy or who had had induced abortions in their history were not excluded All transfused women had had less than four blood transfusions Sera were obtained from blood samples drawn at the time of delivery or within two days after and stored frozen at —20°C for up to 14 days

Lymphocyte suspensions were prepared and stored as previously described (11) All tests were performed as micro lymphocytotoxic tests by the method of *Kissmeyer Nielsen & Kjerbye* (9)

Some definitions

Premature delivery indicates that the child s birth Weight is 2500 g or less

Pre eclampsia indicates that at least two of the three symptoms oedema arterial hypertension and albuminuria are present

Arterial hypertension indicates a blood pressure of more than 140 mm systolic and 100 mm diastolic as measured by use of a mercury sphygmomanometer

Abortion indicates interruption of pregnancy before the end of the 28th week of gestation.

RESULTS

The results of the study are given in the following three tables Furthermore the material comprises four cases of malformations of the infant, two in each group of women No stillbirth is observed in the material The tables show that there were no statistically significant differences between the incidences of the parameters Blood transfusion, pre eclampsia, oedema arterial hypertension, albuminuria premature delivery and abortions, in the two groups of women with and without cytotoxins The frequencies of abortions rise with age and increasing number of pregnancies

DISCUSSION

The incidence of blood transfusion in the history among women with cytotoxins was nearly identical to that of women without

TABLE 1 *Information from Case Reports Blood Transfusion Pre Eclampsia Oedema Arterial Hypertension Albuminuria and Premature Delivery*

	Women without cytotoxins	Women with cytotoxins	χ^2 *	P
Blood transfusion	65 of 878 (7 4 %)	18 of 239 (7 5 %)	0 004	> 0 90
Pre eclampsia	73 of 878 (8 3 %)	21 of 239 (8 8 %)	0 05	> 0 80
Oedema	216 of 878 (24 6 %)	61 of 239 (25 5 %)	0 09	> 0 70
Arterial hypertension	109 of 878 (12 4 %)	25 of 239 (10 5 %)	0 68	> 0 40
Albuminuria	17 of 878 (1 9 %)	6 of 239 (2 5 %)	0 31	> 0 50
Premature delivery	54 of 878 (6 2 %)	16 of 239 (6 7 %)	0 09	> 0 70

* Comparison of the groups of women without cytotoxins and with cytotoxin

TABLE 2 *Abortion in the History in Relation to Number of Pregnancies*

No of pregnancy	Women without cytotoxins	Women with cytotoxins	χ^2 *	P
2	40 of 294 (13 6 %)	10 of 85 (11 8 %)	0 20	> 0 60
3	47 of 118 (39 8 %)	18 of 50 (36 0 %)	0 22	> 0 60
>3	39 of 63 (61 9 %)	17 of 26 (65 4 %)	0 10	> 0 80
Total	126 of 475 (26 5 %)	45 of 161 (28 0 %)	0 12	> 0 90

* Comparison of the groups of women without cytotoxins and with cytotoxins

TABLE 3 *Abortion in the History in Relation to Age Groups*

Age group	Women without cytotoxins	Women with cytotoxins	χ^2 *	P
< 20 years	5 of 78 (6 4 %)	1 of 18 (5 6 %)	0 0002	> 0 90
20 25 years	43 of 376 (11 4 %)	11 of 96 (11 5 %)		
25–30 years	47 of 278 (16 9 %)	14 of 74 (18 9 %)	0 17	> 0 50
30–35 years	17 of 98 (17 3 %)	9 of 29 (31 0 %)	2.57	> 0 10
> 35 years	14 of 48 (29 2 %)	10 of 22 (45 5 %)	1 12 §	> 0 20

* Comparison of the groups of women "without cytotoxins and with cytotoxins"
§ With Yates correction

cytotoxins It was considered that a history of less than four blood transfusions was without importance as regards formation of antibodies during pregnancy This is in accordance with the results reported by *Payne* (13 15) on leuco agglutinins

It is possible from a theoretical point of view that cytotoxins in the serum of the mother might be harmful to the foetus or cause abortion (2 8) *Van Rood* (18) found like *Berah et al* (1) leuco agglutinins as frequently in women with abortions in their history as in women without abortions In a material of 314 pregnant women *Gert Jensen* (4 5) found no correlation between leuco agglutinins and abortions either These observations were emphazised in a later investigation by *Gert Jensen* (6) comprising 883 pregnant women In this study he found an increasing frequency of abortion with increasing number of pregnancies In a material of 574 pregnant women *Terasaki et al* (22) found no relation between the presence of cytotoxins in the current pregnancy and the occurrence of abortions in the history A later work by *Naito et al* (10) supported these results

In this study we found like *Gert Jensen* (7) an increasing frequency of abortion with increasing number of pregnancies and no correlation to the presence of cytotoxins *Naito et al* (10) found the frequency of abortion to be 23 0 per cent in women with cytotoxins and 30 8 per cent in women without antibodies Gert Jensen in his material found the frequencies to be somewhat higher

In this study the frequency of abortion as well as the number of pregnancies rose with increasing age

An estimation whether cytotoxins may induce abortion or whether abortion may provoke the formation of cytotoxins requires an exact anamnestic outline of a great number of clinical facts concerning the abortion Collection of such data was not possible in the present study for want of the relevant information in the case reports Nor have the authors found that all these data have been taken into consideration in other published reports on this subject and therefore it will be very difficult to explain exactly in all cases any causative relationship between cytotoxins and abortions

Gert Jensen (4 5, 6, 7) found a sta

... mature delivery *Payne* (16) and *Berah et al* (1) however found no positive correlation between leuco-agglutinins in serum and premature delivery In the present study the incidence of premature delivery was almost identical in women with and without cytotoxins *Naito et al* (10) found by means of the crossmatch test, a statistically significant correlation between the presence of maternal cytotoxins directed against current foetal lymphocytes and the occurrence of foetal malformations, but no correlation between maternal cytotoxins of unconsidered specificity and foetal malformations current pregnancy

Gert Jensen (6) found no statistically significant correlation between leucoagglutinins and foetal malformations either. The present study comprised four malformed babies. Two in each group. We had only one case with multiple malformations in the group of 239 pregnancies and cytotoxins cannot in general be considered harmful for the foetus as stressed by *Sever et al* (19), who did not use crossmatch tests.

In this study there were no stillbirths and we found no basis for assuming that cytotoxins during pregnancy should cause foetal death (20).

The conclusion drawn from this study must be that cytotoxins do not interfere with the course of the pregnancy, the condition of the pregnant woman, nor do they cause foetal death or malformations.

The authors wish to thank Dr H G Bertelsen Head of the Dept of Obstetrics and Gynaecology Aalborg Hospital North and his staff for allowing the use of case reports and the supply of blood samples.

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Acta path microbiol scand Section B 79 599–605 1971

ANTIBODIES IN BRONCHIAL SECRETIONS FOLLOWING NATURAL INFECTION WITH *MYCOPLASMA PNEUMONIAE*

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The presence of antibodies in sputum specimens of patients with *M pneumoniae* infection and lower respiratory tract illness was investigated Antibodies to *M pneumoniae* were demonstrated by indirect immunofluorescence (IFL) in 44 out of 55 sputa from 29 out of 33 cases The immunoglobulin classes of antibodies in sputa were determined by use of fluorescein conjugated monospecific antisera to IgA IgG and IgM 31 antibody positive sputa were examined with both anti IgA and anti IgG conjugates *M pneumoniae* antibodies og IgA class were present in all these specimens and antibodies of IgG class in 24 of these sputa In addition IgM antibodies were found in 13 out of 27 sputa which also contained IgA antibodies In 5 out of 27 antibody positive sputa examined for antibodies of all three immunoglobulin classes only IgA antibodies were demonstrable Complement fixing antibodies to *M pneumoniae* were present in 17 out of 23 sputa *M pneumoniae* could be isolated from 11 out of 19 sputum specimens which contained specific antibodies

Local immune factors are considered important for protection against respiratory infections (30 25) Resistance to myxovirus and rhinovirus infections has been found to be correlated to the level of antibodies in respiratory secretions rather than to the level of serum antibody (9 27 24) Antibodies to several other viruses and to bacteria have been demonstrated in the secretions of the respiratory tract (for references see 30) These antibodies were associated primarily with 11S IgA which is the predominant immunoglobulin in respiratory secretions (30)

Mycoplasma pneumoniae is an important causative agent of acute respiratory illness (16) often affecting the lower respiratory tract (14, 2) Attempts to detect *M pneumoniae* antibody in nasal secretions have given negative results (28) The present article describes studies demonstrating the presence of *M pneumoniae* antibodies in bronchial secretions of patients naturally infected with *M pneumoniae* The immunoglobulin classes of these antibodies were also determined Part of this work has been described in a preliminary report (3)

Received 29 1 71

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MATERIALS AND METHODS

Sputum and blood specimens were obtained from 33 hospitalized patients with lower respiratory tract illness associated with *M pneumoniae* infection 29 of these patients had pneumonia verified by X ray 25 cases showed a fourfold or greater titre rise of complement fixing (CF) antibodies

that the interferon like substances are produced in response to a secondary infection not associated with the primary neurological disease Naturally, the production of interferon like substances in relation to the pathological process in these patients may also be possible

Further studies, possibly using a more sensitive test system, are required to determine whether the occurrence of interferon like substances in the spinal fluid is a reproducible finding in early stages of multiple sclerosis and other chronic neurological diseases or whether the above observations are merely accidental

A complete review of the results positive as well as negative, obtained in this study of virus inhibiting activity in the various patient groups is in preparation and are to be published later

I am indebted to Dr H Lautrop Head of the Diagnostic Department of the State Serum Institute, Copenhagen, for some of the CSF samples, and to the heads of the clinical departments for their permission to make use of the hospital records for the purpose of this study

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in serum during the course of infection and the remaining 8 cases, from whom only convalescent phase sera were available, had high (≧ 1/128) stationary CF antibody titres to *M. pneumoniae*.

In addition sputa from 10 cases of respiratory infection lacking CF antibodies to *M. pneumoniae* in serum were examined as controls.

Collection and processing of specimens. Sputum was obtained by coughing into sterile cups. The specimens were transported in thermos bottles with ice and were frozen at —65°C until used. 0.1-0.2 ml of the sputum specimen was usually used for isolation of *M. pneumoniae*. The remainder was diluted approximately ½ with phosphate buffered saline (PBS) and homogenized in a tissue grinder followed by centrifugation at 2000 rpm for 30 minutes. The supernatant fluid was used for antibody tests after heating at 56° for 30 minutes and for immunoglobulin determinations unheated. Sputa with gross contamination of blood were not used for antibody tests. The sediments of several sputa were tested for the presence of red blood cells by the guaiac test. A positive reaction was graded 1+, 2+ or 3+. Specimens with 2+ or 3+ reactions were not included in the study. With the guaiac solution used in most instances, a reaction graded as 1+ was found to correspond to a dilution of peripheral blood of 1/5000—1/6000. With another guaiac solution 1+ correspond to a dilution of blood of 1/2000.

Throat swabs were taken for isolation of *M. pneumoniae*. The swabs were transported in tubes containing complete PPLO broth.

Isolation of M. pneumoniae. The procedures used have been described previously (2). The Mycoplasma medium devised by *Hayflick* (15) was used with the addition of penicillin (1000 units/ml) thallium acetate (final dilution 1/2000) and amphotericin (5μg/ml). The broth medium also contained phenol red (0.002 per cent) and glucose (1 per cent). Briefly specimens were inoculated onto agar plates and into diphasic media (agar overlayed with broth). The broth phase was subcultured on agar after about 10 and 30 days. *M. pneumoniae* colonies were recognized by their typical morphology. All isolations were also identified by the ability of the colonies to adsorb guinea pig erythrocytes (8).

Complement fixation (CF) test. The CF antigen was prepared according to the procedure described by *Kenny & Grayston* (18) by chloroform methanol extraction of concentrates of *M. pneumoniae* grown in broth. The CF test was performed with the microtitre system (26) using 2 units of complement, 2–4 units of antigen, 4 units of haemolysin and a 2 per cent suspension of sheep red blood cells. Fixation was allowed to proceed over night.

Tetrazolium reduction inhibition (TRI) test. This method, developed by *Jensen* (17), is based on the ability of growing *M. pneumoniae* organisms to reduce tetrazolium to a red formazan. Specific antisera inhibits the reduction. The procedure followed (29) has been described in detail previously (2).

Immunofluorescence (IFL). The antigen employed in the IFL test consisted of colonies of *M. pneumoniae* transferred from agar blocks to glass slides by melting the agar in hot water as described by *Clark et al.* (6). Approximately 10^5 colony forming units of *M. pneumoniae* strain FH (obtained from dr. R. M. Chanock, NIH, Bethesda, USA) were inoculated onto each agar plate (diameter 5 cm). Colony preparations were made after 4–5 days incubation. Agar blocks with colonies of *M. pneumoniae* were placed on glass slides. The slides were put in a beaker with distilled water at a temperature of 75°C. The water was heated to 80°C whereby the agar blocks fell off. The slides with the colonies were quickly rinsed in distilled water of 90°C. They were airdried and fixed in acetone for 10 minutes. The colony preparations were stored at +4°C and were used in the IFL test within 2–3 days after preparation.

The following fluoresceinlabelled antisera were used. 1) A sheep antihuman gammaglobulin serum reactive with IgG, IgA and IgM prepared and conjugated at Statens bakteriologiska laboratorium (SBL) Stockholm as described by *Bergquist & Schilling* (1). The fluorescein/protein (F/P) weight ratio was 5.0 · 10^{-3}. 2) A monospecific rabbit antihuman IgG serum obtained from dr. *S. G. O. Johansson*, Uppsala and conjugated at SBL (F/P ratio 6,3 · 10^{-3}). 3) Two fluorescein labelled rabbit anti human IgA sera lot nr 305 IV F (F/P 11,2 · 10^{-3}) and 333E (F/P 10,1 · 10^{-3}) purchased from Behringwerke AG. (Marburg Lahn, Germany). 4) A labelled anti human IgM serum (F/P 7,5 · 10^{-3}) from Behringwerke. 5) A fluorescein labelled goat anti human IgM serum lot nr 1387 (F/P 8,6 · 10^{-3}, passive haemagglutination titre against IgM 1/12800, IgG 1/40, IgA 1/40) purchased from Wellcome research laboratories (Beckenham, England). The antibody titre and specificity of the conjugates were tested by double diffusion in gel against IgG, IgA and IgM respectively and by passive haemagglutination of tanned cells coated with these immunoglobulins. The results of these tests for all antisera except anti IgM from Wellcome have been presented in another publication (4). The specificity of the monospecific conjugates was also examined by an IFL performance test against a 19S and 7S fraction of a *M. pneumoniae* convalescent phase serum separated by density gradient centrifugation. The anti IgM conjugates reacted only with the antibodies in the 19S fraction and the anti IgG and anti IgA conjugates only with the 7S fraction.

The indirect IFL staining was performed in the conventional way with incubation at each step for

30 min at room temperature After incubation with the conjugate followed by rinsing in PBS the slides were immersed for 15 minutes in Evans blue diluted 1/10000 in PBS They were rinsed again and then mounted with buffered glycerin

The preparations were examined in a Zeiss fluorescence microscope equipped with a HBO 200 mercury lamp and a dark field immersion condensor using primary filter BG 3 and secondary filters 44 and 47

Quantitative determination of immunoglobulins IgA IgG and IgM concentrations in sputa were determined by the single radial immunodiffusion method essentially as described by *Mancini et al (21)* Rabbit antisera to IgA IgG and IgM respectively were purchased from Behringwerke A pool of sera from 40 blood donors was used as standard The concentration of immunoglobulins in this standard serum pool was determined by dr *S G O Johansson* Uppsala The concentration of IgA was 150 mg/100 ml of IgG 1280 mg/100 ml and of IgM 70 mg/100 ml

The immunoglobulin determinations of some sputum specimens were kindly performed by dr *Renee Norberg* Stockholm

RESULTS

Fifty five sputum specimens from 33 patients with *M pneumoniae* infection were examined for specific antibodies by the indirect IFL technique Antibodies to *M pneumoniae* were detected in 44 sputa from 29 of these cases Sputa from 10 seronegative control cases were negative in the IFL test 31 positive sputum specimens from 20 patients were examined in the IFL test both with anti IgA and anti IgG conjugates *M pneumoniae* antibodies of IgA class were present in all these sputa and antibodies of IgG class in 24 sputa from 17 patients In addition 27 sputa containing IgA antibodies were examined for IgM antibodies Thirteen of these specimens were found to contain IgM antibodies In 5 out of 27 antibody positive sputum specimens examined for *M pneumoniae* antibodies of all three immunoglobulin classes antibodies of IgA class exclusively were demonstrated

Table 1 shows the antibody titres to *M pneumoniae* in sputa and sera and the immunoglobulin levels in sputa of 9 cases

In several instances the amount of sputum was not sufficient for performing all antibody and immunoglobulin determinations

The ratio between the IgA antibody titre in sputum and serum was often higher and in no instance lower than the corresponding ratio for the IgG and IgM antibody titres respectively The antibody titres to *M pneumoniae* were usually considerably lower in sputa than in sera However in two sputa collected 9 and 10 days after onset the IgA antibody titre was 1/4 when the serum antibody titre was as low as 1/8

No sputum specimens were obtained later than 8 weeks after onset of illness In the three sputa collected 7 8 weeks after clinical onset *M pneumoniae* antibodies were still demonstrable

Several sputa were examined for *M pneumoniae* antibodies also by the CF test which measures antibodies of IgM and IgG class but not of IgA class 17 out of 23 sputa were positive for CF antibodies The results of some of these tests are shown in Table 1

Only a few sputa were examined for TRI antibodies since most patients had been treated with tetracycline or erythromycin and the presence of these antibiotics in sputa and sera may give false positive reactions in the TRI test Three of six sputa examined gave a positive reaction in the TRI test, but 2 of the positive specimens had been collected during tetracycline treatment

The serum antibody titres presented in table one illustrates the sensitivity of the IFL test compared with the CF and TRI methods It appears that in most sera the titres of IFL antibodies of IgG class were as high as or higher than the corresponding CF and TRI antibody titres

Some sputa included in the study gave a weak (1+) positive reaction in the guaiac test indicating traces of blood in the specimen However a guaiac reaction graded as 1+ was found to correspond to an approximate dilution of peripheral blood of 1/2000–1/6000 and it is unlikely that this small contamination of blood can have contributed to the antibody content in sputum

For quantitation of IgA in sputum a serum

TABLE 1 *Antibody Titres in Sputa and Sera measured by Immunofluorescence (IFL), Complement Fixation (CF) and Tetrazolium Reduction Inhibition (TRI) Tests and Immunoglobulin (Ig) Levels in Sputa of Cases with* M. pneumoniae *Infection. The Titres Are Expressed as the Reciprocal of the Dilution. nd = Not Done*

Case	Days after onset of illness	Specimen	Guaiac test	Antibody titre to *M. pneumoniae* IFL IgA	IFL IgG	IFL IgM	CF	TRI	Ig levels mg/100 ml IgA	IgG	IgM	Isolation of M. pn
181	14	sputum	—	8	<2	<2	<2	nd	11	<6	<8	+
	14	serum		256	256	128	128	128				
581	10	sputum	nd	<2	<2	<2	nd	nd	20	10	<5	—°
	10	serum		<8	32	nd	16	<2				
	46	sputum	nd	8	4	<2	nd	nd	15	5	<5	—°
	46	serum		128	512	nd	128	256				
660	14	sputum	+	8	4	2	4	nd	45	25	10	+
	14	serum		256	1024	≥128	128	nd				
	19	sputum		8	4	<2	4	8*	30	16	<8	—
	19	serum		256	512	nd	256	256*				
703	15	sputum	+	8	2	<2	nd	nd	30	30	10	+°
	15	serum		128	128	128	128	128*				
	22	sputum	—	8	<2	<2	nd	nd	10	5	<5	—°
	22	serum		256	512	512	nd	256*				
	37	sputum		8	<2	nd	<4	nd	10	5	<5	—°
	37	serum		128	256	nd	256	128				
773	11	sputum	+	16	16	16	8	<2	55	35	10	+
	11	serum		64	128	256	128	16				
	42	sputum	—	16	nd	nd	nd	nd	nd	nd	nd	nd
	42	serum		128	512	256	512	256				
793	19	sputum	—	32	8	≥2	nd	nd	28	24	nd	+°
	19	serum		128	1024	512	256	nd				
	55	sputum	—	4	<2	<2	nd	nd	19	19	<5	nd
	55	serum		256	1024	256	128	32				
822	24	sputum	+	32	8	16	nd	nd	48	24	14	—°
	24	serum		512	512	1024	512	512*				
	34	sputum	—	8	2	2	2	nd	12	6	<8	—°
	34	serum		128	256	256	512	nd				
861	9	sputum	—	4	<2	<2	<2	nd	22	10	<10	—
	9	serum		8	16	8	16	<2*				
	49	sputum	—	1	<1	<1	<1	nd	5	<5	<5	—
	49	serum		256	256	256	256	16				
854	22	sputum	+	32	16	32	nd	nd	32	33	8	+
	22	serum		256	512	512	2048	256				
	58	sputum	—	2	2	2	nd	nd	7	<6	<7	nd
	58	serum		128	1024	512	1024	128				

* specimens collected during treatment with tetracyclines
° throat swab specimen was used for isolation attempts

7S IgA was used as standard in the immuno diffusion test which gives too low values for secretory 11S IgA (30) The IgA levels in sputa (Table 1) would probably have been at least 3 times higher if an 11S IgA preparation had been used as standard

Isolation of *M pneumoniae* was attempted from 19 sputum specimens which contained IFL antibodies *M pneumoniae* was recovered from 11 of these specimens In 18 additional cases isolation was attempted from throat swabs collected on the same occasion as sputa positive for antibodies The microorganism was isolated from 5 of these throat swabs

DISCUSSION

The presented data show that specific antibodies are present in bronchial secretions in most cases of *M pneumoniae* infection with lower respiratory tract illness

Sputum was chosen for examination of antibodies rather than nasal washing which is the source of material most commonly used for studies of local antibody to respiratory pathogens since all cases available for study had lower respiratory tract illness and since previous attempts to detect *M pneumoniae* antibodies in nasal secretions by the TRI test had been negative (28)

The indirect IFL technique appears to be the method of choice for the demonstration of *M pneumoniae* antibodies in respiratory secretions since it measures antibodies of all the three main immunoglobulin classes and permits the determination of the immunoglobulin class of the antibodies without previous fractionation of the specimens *Taylor Robinson and associates* (29) found the IFL technique to be more sensitive than CF TRI and indirect haemagglutination tests when sections of *M pneumoniae* infected chicken embryo bronchi were used as antigens (20) IFL employing colonies of agar transferred to glass slides as antigens has been considered an insensitive serologic method (5 16) However in our hands this latter technique gave serum antibody titres as high as or higher than the CF and TRI tests (see Table 1) *Lind* (19) has also demonstrated high antibody titres in human *M pneumoniae* antisera using colonies as antigen in the IFL test The counterstaining with Evans blue used in the present study giving a red background colour to the colonies reduced the nonspecific staining and made it easy to distinguish the green positive staining of the colonies even when the positive reaction was weak (for colour photos of positive and negative IFL reactions with *M pneumoniae* colonies see 4)

In IFL studies of antibodies to certain bacterial antigens it has been observed that excess of IgG antibodies may block the reaction of IgM and IgA antibodies (7 23) This blocking phenomenon did not seem to seriously interfere with the measurement of IgA and IgM antibodies to *M pneumoniae* since the titres of these antibodies in serum were almost as high as the IgG antibody titre and in sputa the IgA antibody titre was often highest It should be noted that it was necessary to use anti IgA conjugates with a very high F/P ratio in order to obtain optimal IgA antibody titres to *M pneumoniae (4)*

Antibodies found in respiratory secretions following infection or immunization with respiratory viruses have been associated mainly with IgA (30 24) There is evidence that secretory IgA antibody is synthesized locally in plasma cells of the mucosa (30) However it is known that bronchial inflammation may cause increased capillary permeability resulting in transudation of serum antibody into respiratory secretions (10 30) This phenomenon has been called pathotopic potentiation of immunity (10) It is likely that part of the *M pneumoniae* antibodies in sputa were derived from serum by this mechanism since most sputa examined contained both IgA and IgG antibodies and half of the specimens also IgM antibodies However the fact that only IgA antibodies were demonstrated *in some* sputa and that the ratio between the sputum and serum antibody *titres often* was higher for IgA than for IgG and IgM antibodies suggest that locally

produced IgA antibodies contributed to the antibody content in sputa

The possibility also exists that part of the IgG and IgM antibodies present in sputa may have been produced locally since IgA, IgG and IgM producing cells have been found in the normal bronchial mucosa in the approximate ratio of 5 5 1 (22)

It is reasonable to assume that antibodies appearing in bronchial secretions as a result of transudation and/or local production participate in the defense against *M pneumoniae* in the respiratory tract It was evident though, that *M pneumoniae* organisms could persist in the respiratory tract in the presence of specific antibodies in bronchial secretions

In the hamster experimental model local immunity appears to be important for resistance to *M pneumoniae* induced pneumonia (11, 12, 13) Thus, hamsters previously infected with *M pneumoniae* were found to be more resistant to pneumonia upon challenge, even if they had low serum antibody titres, than parenterally vaccinated animals with high serum antibody titres (11) However, antibodies to *M pneumoniae* as measured by growth inhibition (GI) were detected in bronchial washings of challenged animals only (13) In further experiments bronchial washings of infected non vaccinated hamsters also were found to contain GI antibody, although in very low titre (12) In the discussion of these results *Fernald* (12) considered the possibility that cellular immune mechanisms also may contribute to the local defense against *M pneumoniae*

The role of the antibodies in bronchial secretions for protection against respiratory illness caused by *M pneumoniae* in man remains to be determined This matter may be elucidated by studies of antibodies in serum and secretions in relation to resistance in volunteers vaccinated and challenged with *M pneumoniae*

The skilled technical assistance of *Lana Ronnerfors* and *Karin Knutsson* is gratefully acknowledged

This work was supported by the *Swedish Medical Research Council* (Project No B69-40X-2380-02 and B71-16X-2380-04)

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A complete review of the results, positive as well as negative, obtained in this study of virus inhibiting activity in the *various patient groups is in* preparation and are to be published later

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Acta path microbiol scand Section B 79 609–614 1971

NEISSERIA GONORRHOEAE. COLONY VARIATION I

A E JEPHCOTT* and ALICE REYN

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Five characteristic types of gonococcal colonies have been recognized These and the special optical system needed to differentiate them are described in detail Certain types were shown to be associated with clinical materials whereas others arose on laboratory subculture The authors suggest that any work relevant to clinical infection (except sensitivity testing) or the aggressive action of gonococci should be carried out on clinically associated types

Gonococcal research is much complicated by incomplete knowledge of the changes which take place when gonococci are subcultured Results obtained with old cultures may not necessarily apply to newly isolated strains hence a marker which will readily identify unaltered gonococci is needed It has been suggested that this is now available (8)

Many properties of gonococci are known to alter after subculture These include nutritional and CO requirements antigenic structure (2 7 15 16) infectivity for the male urethra (8 11) ability to withstand killing factors in human serum (17) and colonial morphology

In practice the most convenient marker is change in colonial appearance This was reported by *Lipschutz* (10) in 1904 and was described in greater detail by *Hill* (5) in 1946

Colonial variation also seems to accompany other changes in the gonococcus Thus already in 1898 differences in the colonies from acute and chronic gonorrhoeal infections had been noticed (18), and from *Atkin's* (1) work in 1925 it became apparent that this variation was the same as that which occurred on subculture He also considered that it was accompanied by an antigenic change and speculated on a concurrent loss of virulence

Subsequently *Casper* (3 4) and *Hill* (6) echoed this concept and emphasized the possibility of loss of virulence although *Mahoney et al* (11) were unable to verify this experimentally

Kellogg et al (8 9) have recently made a detailed study of the problem They described four characteristic types of colonies (1 to 4) and showed that types 1 and 2 grew in primary cultures from acute infections, whereas types 3 and 4 arose on subculture *Sparling & Yobs* (14) from the same laboratory, confirmed the association of types 1 and 2 with acute human infection *Kellogg et al* (8, 9) showed that organisms of types 1 and 2 were capable of setting up infection in the human urethra whereas those of types 3 and 4 were

Received 9 ii 71

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Studies carried out in Copenhagen during visits by *A E Jephcott* financed by the *Wellcome Trust* and the *World Health Organization*

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that the interferon like substances are produced in response to a secondary infection not associated with the primary neurological disease Naturally, the production of interferon like substances in relation to the pathological process in these patients may also be possible

Further studies, possibly using a more sensitive test system, are required to determine whether the occurrence of interferon like substances in the spinal fluid is a reproducible finding in early stages of multiple sclerosis and other chronic neurological diseases or whether the above observations are merely accidental

A complete review of the results, positive as well as negative, obtained in this study of virus inhibiting activity in the various patient groups is in preparation and are to be published later

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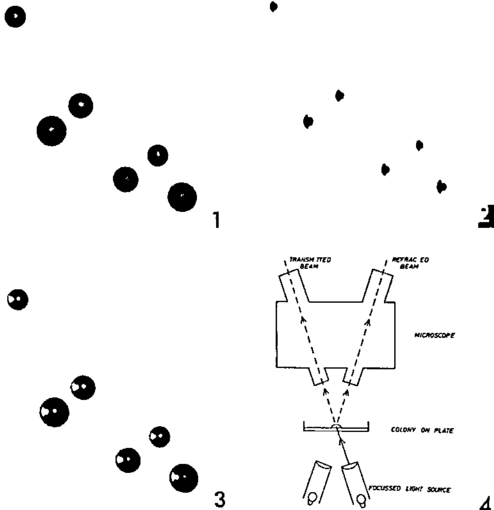


Fig 1 Transmitted light Type 2 colonies Strain F62

Fig 2 Refracted and reflected l ght Same colonies as in Figs 1 & 3

Fig 3 Composite image Same colonies as in Figs 1 & 2

Fig 4 Diagram of light paths One lamp illuminated only

hemispherical colonies which tended to revert to more usual forms on subculture

Examination of the colony types present in each of 265 swabs from clinical cases (mainly acute) confirmed that type 1 or type 2 nearly always predominated, and that only in less than 1 per cent did type 3 or type 4 do so Like Sparling & Yobs (14) we found that in about 10 per cent of cases the predominant colony could not be typed (Table 1)

Twenty old laboratory strains were also examined, only colonies of types 3, 4 and 5 were revealed in 18 of these In one culture a few type 1 colonies were present, and in another significant numbers of type 1 and 2 colonies remained

40*

not They also detected metabolic differences between different types of the same strains of gonococcus

Thus colonial appearance seems to offer a convenient marker for many changes that occur in the subcultured gonococcus

The present investigation was undertaken in an attempt to corroborate these observations and in a subsequent study the shapes of the characteristic colony types were deduced from the profiles of vertical sections through the colonies (13)

MATERIAL AND METHODS

The *medium* was that described by *Kellogg et al* (8) It was made from Difco GC Medium Base with a 2 per cent supplement of ferric nitrate, dextrose, L glutamine and co carboxylase After growth on this medium for 16–18 hours in a 10 per cent CO_2 atmosphere all colonies appeared similar to the naked eye They could be differentiated only by their differing abilities to refract and reflect light under the microscope This made the conditions of viewing the colonies of paramount importance

Several *lighting systems* were tried, including the diffused oblique beam of transmitted light, described by *Kellogg et al* (8), but optimal differentiation of the colony types could be secured only when stereoscopic microscopes with double systems of substage lighting were used

These instruments were set up so that one beam of light was directed through the colony into each objective The resulting image at each eye piece was a composite one It consisted partly of light from one source, transmitted directly through the colony, and partly of light from the other source that had been refracted and reflected by the colony The two components could be demonstrated separately by switching off one of the substage lights when the transmitted picture would appear in one eye piece and the refracted picture in the other

Fig 1 shows the transmitted light picture i e the light that had been transmitted directly through the colonies Fig 2 shows the refracted light picture, i e the light that had been reflected and refracted by the colonies Fig 3 shows the combined picture as it appeared to the observer Fig 4 illustrates the light paths described For simplicity, light from only one of the pair of light sources is shown

About 300 fresh *strains* and 20 old laboratory strains were examined including strain F62 (*Kellogg)* The latter had been selectively transferred by *Kellogg* for about three years to high yield of type 1 colonies

RESULTS

All four colony types were recognizec the illumination system used, they app as described below

Type 1 These were small, dark golden colonies with discrete edges The refrac components produced a highlight on eac side, which were pronounced but soft edged Colonies were easily emulsified but formed rough suspensions in saline (Fig 5)

Type 2 These were slightly smaller, darker colonies Their edges were very clearcut indeed The highlights were bright and sharp edged The colonial surface was very shiny Colonies were not easily emulsified but broke into large fragments Suspensions were markedly rough in saline (Figs 3 and 6)

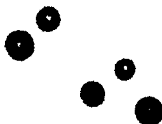
Type 3 These were larger and flatter than types 1 or 2 and had a dull brown granular appearance Their edges were smooth or finely serrated and the highlights were absent The colonies were soft but still formed rough suspensions in saline Colonies from one strain of gonococcus would appear lighter or darker than those from another, and on some occasions darker and lighter colonies could be demonstrated in the same strain (Fig 7)

Type 4 These were similar in size and shape to type 3, but non granular and almost colourless Their edges were entire and the highlights absent These colonies were very easily emulsified and formed smooth suspensions in saline (Figs 5, 7 and 8)

Not all colonies seen fitted these descriptions

Another type—which we named *Type 5*—was seen first in an old laboratory strain but was later found in other strains This was very shiny like the type 2 colony but was as large as the type 3 It was made up of very obvious granules and was dark brown in colour Frequently a series of concentric ri could be seen on the surface The edge wa coarsely irregular (Fig 8)

Certain strains gave rise to small granula







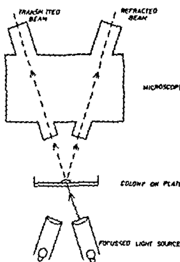



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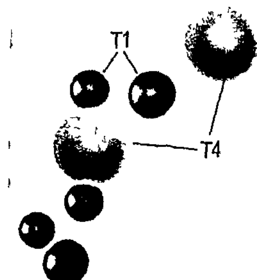
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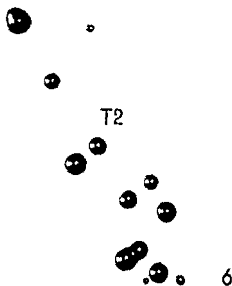
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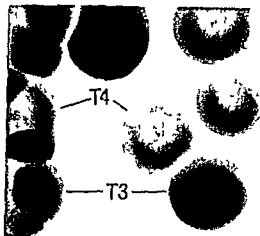
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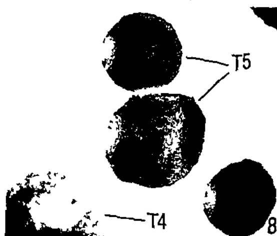


Fig 5 Types 1 & 4 colonies Strain F62

Fig 6 Type 2 colonies Strain F62

Fig 7 Types 3 & 4 colonies Strain F62

Fig 8 Types 4 & 5 colonies Strain 43562/SS 1944

Subculture of examples of the different colonies showed that whereas most daughter colonies were of the parents' type, some would appear as other types We noted all possible changes between types 1 2, 3 and 4 but the most common changes were from type 1 to type 4, and from type 2 to type 3 As the reverse changes were much less common, unless a deliberate selection of colonies was made, a population of type 1 or type 2 colonies rapidly became one of type 3 or type 4 Colonies which were kept in one colony type through many generations tended to become more "fixed' and less liable to change type, whereas fresh strains rapidly altered type

To demonstrate this tendency of cultures to "degenerate' into types 3 and 4 on subculture, pure cultures of each colony type of representative strains were sequentially subcultured daily for eleven days No effort was made to select colonies of a particular type

TABLE 1 *Predominating* Colony Types of Neisseria gonorrhoeae Isolated from Clinical Material*

Colony type	Number of specimens	Percentage of total
1	169	63 7
1+2	6	2 3
2	56	21 1
3	1	0 4
4	1	0 4
Unidentified	32	12 1
Total	265	100 0

* Predominating means ≧ 70 per cent in type specified

at each subculture, but the relative proportion of the initial type present in each culture each day was recorded The rate of change varied from strain to strain but the overall picture presented was always the same Tables 2 and 3 list the results for a typical strain They show that the clinically associated types 1 and 2 were replaced by laboratory associated types 3 and 4, with type 2 disappearing the more rapidly They also show that types 3 and 4 interchanged between themselves but did not give rise to significant numbers of types 1 or 2

The antibiotic sensitivities of all available colony types of six strains of gonococci were determined No differences were found within a given strain

DISCUSSION AND CONCLUSIONS

Much previous work suggests that gonococci change on subculture In other Gram negative bacteria *e g Haemophilus influenzae* (12), change from the virulent to a non virulent form occurs on subculture, and is associated with change in colonial morphology A similar phenomenon has been demonstrated for *N gonorrhoeae* by *Kellogg* (8, 9)

We have examined many cultures of gonococci over long periods and confirm that several characteristic gonococcal colony morphologies are recognizable, and that these can be separated into the clinically associated types, (1 and 2) and laboratory associated types (3, 4 and 5) From this it must follow that the gonococci of the colony types 1 and 2 correspond more closely than do those of the other types to the state of the organisms as they occur in the acute case

Kellogg described four types, and we have added a highly characteristic fifth type, but colonies can assume other morphologies We have seen a variety of granular forms which did not resemble any of the five types Similarly, non characteristic colonies were mentioned by *Kellogg* (8, 9) and by *Sparling & Yobs* (14) These forms can be due to media variations (8 9), but we have observed that they can also occur on plates capable of supporting characteristic forms Such colonies form a minority of primary cultures and can occur on subculture of established clones They tend to revert to more characteristic appearances on further subculture and have not produced any real difficulties

It should be added that in our experience the antibiotic sensitivities of gonococci do not alter on subculture Thus no variation may be expected to accompany any change in colony type Our present results bear this out *Kellogg et al* (8, 9) made similar observations

TABLE 2 *Distribution of Colonial Types during Eleven Daily Subcultures of Strain F62*

Original colony type	Percentage of Original Colony Type Present on Successive Days 1	2	3	4	5	6	7	8	9	10	11
Type 1	98	50	30	30	20	20	15	5	10	1	0
Type 2	90	95	50	10	0	1	0	0	0	0	0
Type 3	98	98	95	95	90	80	75	80	50	50	10
Type 4	95	98	90	98	90	95	80	80	80	70	80

TABLE 3 *Percentage of Each Colony Type Present on Day Eleven Same Experiment as in Table 2*

Original colony type	Type 1	Type 2	Type 3	Type 4
Type 1	0	0	10	90
Type 2	0	0	60	40
Type 3	0	0	10	90
Type 4	0	0	20	80

It is now possible to recognize an *in vitro* change in *Neisseria gonorrhoeae* by inspection of the colonies Further, it appears that other changes take place at the same time As this is the only practicable test available at the moment we suggest that any work relevant to clinical infection (except sensitivity testing) or the aggressive action of gonococci should be carried out on clinically associated types

Mrs *R Glarborg* and Mrs *L Olesen* are thanked for valuable technical assistance

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Acta path microbiol scand Section B 79, 615 619, 1971

SEROLOGICAL PROPERTIES OF LIPOPOLYSACCHARIDE FROM STRAINS OF ORAL *VEILLONELLA*

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Lipopolysaccharide (LPS) extracted with phenol water from five oral strains of *Veillonella* was studied by indirect haemagglutination techniques The highly active LPS showed sero logical type specificity LPS from at least two of the strains contained more than one antigenic determinant carried by the same molecular complex The determinant groups were of carbohydrate nature Inhibition of haemagglutination with mono and disaccharides gave information of the chemical composition of the determinant group in LPS from one of the strains examined The LPS preparations were immunogenic in rabbits, giving rise to both ME resistant antibodies and antibodies sensitive to reductional cleavage with ME

The chemical composition of phenol water extracted lipopolysaccharide (LPS) from whole cells of four strains of oral *Veillonella* was recently examined in some detail (3) The overall chemical composition resembled that of LPS from Gram negative aerobic bacilli (5) LPS from all strains contained 2 keto-3-deoxyoctonate (KDO), heptose, glucosamine, galactosamine and glucose Additional sugars present were galactose and ribose in LPS from one strain, and galactose in LPS of another of the four strains examined The isolated LPS differed also with respect to predominant sugar released by mild acid hydrolysis

The serological properties of the isolated LPS have been studied by indirect haemagglutination techniques In particular, the aim of the study was to verify, if possible, the serological type-specificity of LPS from these bacteria (6), and to investigate whether serological multispecificity could be demonstrated in such preparations

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MATERIALS AND METHODS

The catalase positive *Veillonella* strains Ve5 and Ve8 and the catalase negative strains Ve9, Ve10 and Ve11 were isolated from saliva of five human, adult subjects Cultivation of strains and preparation of LPS have been described *earlier* (3) Treatment of LPS with periodate or with *pronase* (B-grade, Calbiochem, Los Angeles, California)

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[illegible] treatment of *antiserum* with 2 mercapto ethanol (ME) was performed as described previously (2)

Sensitization of sheep erythrocytes for indirect haemagglutination was performed with LPS which had been *treated with NaOH* for 1 hour at 56° C, neutralized with HCl, and dialysed against 0 007 M phosphate buffered saline pH 7 2 (2) Unless *otherwise stated* a 1 per cent suspension of sheep cells were sensitized with an equal volume of LPS

Acta path microbiol scand Section B 79, 620 634, 1971

ANTIBODY RESPONSES IN *MYCOPLASMA PNEUMONIAE* INFECTION IN RELATION TO SERUM IMMUNOGLOBULINS, ESPECIALLY IgM

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Comparison of the antibody response to *M pneumoniae* in patients with pneumonia of different ages showed that most cases below 20 years had an early predominance of IgM relative to IgG complement fixing (CF) antibodies whereas most adults above 40 years possessed more IgG than IgM CF antibodies Young patients usually developed higher titres of CF than of tetrazolium reduction inhibiting (TRI) antibodies while older patients often showed higher TRI than CF antibody titres 7 out of 10 adult patients with excretion of *M pneumoniae* of short duration formed little or no IgM antibodies while most cases with prolonged excretion had a rather potent IgM antibody response in the early phase of infection But a longlasting persistence of *M pneumoniae* in the throat was not necessarily accompanied by a longlasting IgM antibody response The total serum IgM level increased during infection in patients who developed high or moderate titres of IgM antibodies to *M pneumoniae* and of cold agglutinins but showed little or no change in patients who formed *M pneumoniae* antibodies of IgG class almost exclusively Absorption with *M pneumoniae* organisms of 8 sera with elevated IgM resulted in a 8–34 per cent reduction of total IgM Removal of the cold agglutinins reduced the IgM concentration by 11 per cent in one serum but had little or no effect on the IgM level in 7 other sera Absorbable *M pneumoniae* antibodies and cold agglutinins accounted for 30–57 per cent of the IgM increase which was demonstrated during the infection Two to four years after *M pneumoniae* pneumonia, CF antibodies persisted in 38 out of 41 cases TRI antibodies in 39 of 41 cases and IFL antibodies of IgG class in all of 41 cases 50 per cent of the sera obtained 4 years after infection contained IgM antibodies demonstrable by IFL and CF

Antibodies to *M pneumoniae* can belong to the three main immunoglobulin classes IgM (19S) IgA and IgG (7S) (3, 7, 16) Previous studies (3) showed that in most cases of *M pneumoniae* infection complement-fixing (CF) and tetrazolium reduction inhibiting (TRI) antibodies of 7S class increased relative to antibodies of 19S class with time after infection Indirect haemagglutinating antibodies (IHA) were predominantly 19S globulins However, there was considerable individual variation in the ratio of 19S and 7S antibodies even when compared at approximately the same time after onset of illness In the present work the immune response was related to the age of the patients and the

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duration of excretion of *M pneumoniae* The persistence of antibodies of different molecular classes several years after infection was also examined Furthermore the occurrence of *M pneumoniae* antibodies in individuals of different ages without respiratory illness was determined

The level of IgM in serum has been found to increase during infection with *M pneumoniae* (15, 30, 31) In the present work the IgM levels were studied in relation to the immunoglobulin classes of the antibodies formed during the infection Absorption experiments were performed to determine the contribution of *M pneumoniae* antibodies and cold agglutinins to the raised IgM levels

MATERIALS AND METHODS

Throat and blood specimens were obtained from hospitalized patients with pneumonia and from family contacts with pneumonia or febrile bronchitis (4) Repeated specimens were collected from the patients usually during a period of several months or years The demonstration of a fourfold change in antibody titre or of high stationary antibody titres (≧1/64) in combination with isolation of the micro-organisms was considered evidence of current or recent *M pneumoniae* infection

Sera were also obtained from 782 individuals presumed not to have acute respiratory illness 573 of these sera had been collected during 1966 and 1967 from 294 men and 279 women of different ages between 20 and 79 years as part of a health control study in the Stockholm area The remaining 209 sera (obtained from Dr *G Huldt*) were from subjects of different ages below 20 years and had been sent to the laboratory during 1969 for examination of Toxoplasma antibodies

Media The medium devised by *Hayflick* was used (19) It contained 7 parts Difco PPLO agar or broth 2 parts not heated horse serum 1 part 25 per cent yeast extract penicillin 1000 units/ml and amphotericin 5 μg/ml 1 per cent glucose and 0 002 per cent phenol red were added to broth medium Media used for isolation also contained thallium acetate (1/2000 final dilution)

Isolation of M pneumoniae The procedures used have been described previously (4) Two throat specimens a throat swab and a sputum or a throat washing were usually collected on each occasion Both agar plates and diphasic medium were used for isolation *M pneumoniae* colonies were recognized by their typical morphology All isolations were also identified by the ability of the colonies to adsorb guinea pig erythrocytes (13)

Growth of M pneumoniae for antigen preparation In the early phase of the studies *M pneumoniae* organisms (strain FH, obtained from Dr *R M Chanock*, NIH Bethesda) were grown in broth under continuous shaking and harvested as described previously (2) In subsequent studies though the organisms were allowed to grow on the glass surface of a Povitsky bottle containing 500 ml of broth (34) After incubation at 37°C for 3–4 days the broth was discarded the organisms attached to the glass surface were washed 3 times with phosphate buffered saline (PBS) and then removed by shaking the bottle with sterile glass beads in PBS The suspension of organisms was centrifuged at 15000 g for 30 minutes and then resuspended in 5 or 10 ml PBS This concentrated suspension of *M pneumoniae* was used for preparation of CF and IHA antigens and for absorptions

Complement fixation (CF) test The CF antigen was prepared by chloroform methanol extraction and KCl partition of a concentrated suspension of *M pneumoniae* according to the procedure described by *Kenny & Grayston* (21) The CF test was performed with the microtitre system (32) using 2 units of complement, 2–4 units of antigen 4 units of haemolysin and a 2 per cent suspension of sheep red blood cells Fixation was allowed to proceed overnight

Indirect haemagglutination (IHA) test The procedure described by *Lind* (24) employing formalinized erythrocytes for sensitization with *M pneumoniae* antigen was essentially followed as has been described in detail previously (4) A concentrate of *M pneumoniae* organisms sonicated in a MSE 50 W ultrasonic disintegrator for 30 minutes was used for sensitization of erythrocytes Heated sera (56° 30 minutes) were titrated with the microtitre system in disposable plastic plates and an equal volume of 1 per cent of sensitized erythrocytes was added The plates were read after incubation for 2 hours at 28°C The sera also were tested against nonsensitized formalinized cells and if reactive with such cells they were retested after absorption for 10 minutes at room temperature with 0 1 ml packed sheep erythrocytes per 0 1 ml serum

Tetrazolium reduction inhibition (TRI) test This test developed by *Jensen* (20) was performed as described by *Taylor Robinson et al* (37) Twofold dilutions of previously heated sera starting with dilution ½ were made in 0 025 ml volumes in broth medium containing 0 05 per cent triphenyltetrazolium chloride 0 025 ml of *M pneumoniae* diluted in the same medium so as to contain approximately 10^3 colonyforming units/0 025 ml was added to each cup followed by 0 15 ml of the same medium The plates were sealed with plastic tape and incubated at 37°C During growth of *M pneumoniae* the tetrazolium is reduced to

a red formazan This usually occurred after 5 days The highest serum dilution which prevented a colour change was taken as the titre of inhibiting antibodies The titration of two reference convalescent sera were included in each test

The presence of antibiotics (tetracyclines or erythromycin) in serum may give false positive reactions

Cold agglutinin (CA) test (10) Twofold serum dilutions were mixed with an equal volume of a 0,2 per cent suspension of human ORh- erythrocytes and the mixture was incubated at 4°C over night Titres were expressed as the serum dilution — prior to addition of erythrocytes — of the last tube showing agglutination visible to the unaided eye A fourfold increase in titre or a titre of ≧1/64 was regarded as positive

Density gradient ultracentrifugation A gradient of sucrose ranging from 10 to 37 per cent was prepared 0,4 or 0,6 ml of previously heated serum diluted ½ or ¼ in PBS was layered over the gradient, final volume of 5 ml, which was then centrifuged in a Spinco centrifuge using a SW 39 rotor at 35000 rpm for 18 hr at 5°C Serial fractions of 0,4 to 0,5 ml were collected dropwise from the bottom of the tubes All fractions from each serum were tested for antibody activity on the same occasion The sum of the antibody titres of the fractions in the 19S and 7S regions was calculated separately to obtain the total antibody activity associated with each of these immuno globulins The relative amount of 19S and 7S antibody was then expressed in per cent of the total recovered antibody activity

The 7S fractions contained both IgG and IgA as shown by immunodiffusion tests CF antibodies in the 7S fractions could only be of IgG class since IgA antibodies do not fix complement

Quantitative determinations of IgM and transferrin The concentration of IgM and transferrin in sera was determined by the single radial diffusion method essentially as described by *Mancini et al* (27) Rabbit antisera to IgM and transferrin respectively were purchased from Behringwerke A pool of sera from 40 blood donors, stored in small aliquots at —20°C, was used as standard IgM values were expressed in per cent of this standard The IgM content of the standard serum pool was 70 mg/100 ml, kindly determined by Dr *S G O Johansson*, Uppsala

Absorption experiments Human immune sera to *M pneumoniae* which contained cold agglutinins and showed increased levels of IgM were absorbed with *M pneumoniae* antigen and with human ORh- erythrocytes in the cold

Non heated sera were diluted ¼ to $^1/_8$ depending on the IgM content, and were divided in 3 portions marked a, b, c a served as unabsorbed control, b 0,5 ml of diluted serum was absorbed with the pellet obtained by centrifugation of 0,5 ml of a suspension of *M pneumoniae* organisms (see preparation of *M pneumoniae* antigen) The mixture was kept for 1–2 hours at room temperature and then at +4° over night before being centrifuged at 15000 r p m (*Spinco centrifuge, rotor 65*) for 30 min The supernatant was collected and absorbed once more c1 1 ml of diluted serum was absorbed twice with 0,5 ml of packed human O erythrocytes for 5–18 hours at +4°C The supernatant obtained after centrifugation of the mixture at 3000 r p m in the cold was divided in two aliquots 0,5 ml of the diluted serum absorbed with red blood cells was further absorbed twice with *M pneumoniae* antigen as described under b = sample c2

The samples a, b, c1 and c2 were examined with regard to IgM content (non heated) and antibody activity (after heating at 56° for 30 min)

The dilution which had occurred during the absorptions was estimated by measuring the decrease in the transferrin level after the absorptions by the radial immunodiffusion technique

Immunofluorescence (IFL) The procedure used has been described in detail previously (7) The antigen employed in the test consisted of colonies of *M pneumoniae* transferred from agar to glass slides (9) Two fluoresceinconjugated antisera were used 1) a rabbit anti human IgG serum obtained from Dr *S G O Johansson*, Uppsala, conjugated in this laboratory to a fluorescein/protein (F/P) weight ratio of $6{,}3 \times 10^{-3}$ and employed in dilution $^1/_8$, 2) a sheep anti human IgM serum purchased from Wellcome research laboratories Beckenham, England (F/P ratio $8\,6 \times 10^{-3}$) used in dilution $^1/_{20}$

Details about the titre and specificity of these antisera, the staining procedure and the fluorescence microscope used are found elsewhere (6, 7)

RESULTS

19S (IgM) and 7S antibody response in relation to duration of excretion of M pneumoniae Sera from 7 patients with excretion of *M pneumoniae* during the first week of illness only and from 3 patients with a fourfold or greater rise in CF antibody titre to *M pneumoniae* but without demonstrable excretion of the micro organism were fractionated into 19S and 7S immunoglobulins by density gradient centrifugation (Table 1) 8 out of these 10 cases had been treated with tetracyclines 5 out of 10 patients were found to have CF and TRI antibodies of 7S class exclusively

TABLE 1 *Antibody Pattern in Relation to Excretion of* M pneumoniae *in 30 cases of* M pneumoniae *Infection 10 20 Days after Onset of Respiratory Illness*

Duration of excretion	No of cases	CF			TRI			IHA			CA	
		>50 % 19S	>50 % 7S	only 7S	>50 % 19S	>50 % 7S	only 7S	Pos titre ≥64	Pos titre <64	Neg	titre ≥64	titre <64
No excretion or less than 8 days	10	1*	4§	5		1	6	1	3	5	3	7
4 to 20 weeks	20	11†	6	3	5	6	3	19		1	17	3

* One case, § Two cases, † Six cases: not examined for class of TRI antibodies

already in the earliest available serum with demonstrable antibodies collected 10–20 days after onset of illness Only one of the remaining five cases had a predominance of 19S over 7S CF antibodies Titres of IHA antibodies which are mainly IgM, were negative or low (<1/64) in 8 out of 9 examined cases and cold agglutinins which are only IgM were present in only 3 of the 10 cases Thus 7 of these cases with short time excretion or without demonstrable excretion (all of them examined by repeated throat cultures) formed little or no IgM antibodies in response to the infection It should be noted that all 10 cases in this group were adults more than 20 years old In contrast, all of 33 patients in the 5–19 year age group who were examined by repeated throat cultures were found to excrete *M pneumoniae* for more than 8 days after onset of illness

Examination of sera from 20 patients 6 of them below 20 years with shedding of *M pneumoniae* for 4 to 20 weeks after clinical onset in spite of treatment with tetracyclines (in 17 cases) showed a varying pattern with regard to the distribution of antibodies within the immunoglobulin classes (Table 1) 11 of these 20 cases had a preponderance of 19S relative to 7S CF antibodies in the early convalescent phase sera TRI antibodies of 19S class predominated in 5 out of 14 cases examined for such antibodies (The 6 cases not examined for TRI antibodies belonged to the group of cases which had CF antibodies predominantly of 19S class) IHA antibodies in titre >1/64 appeared in 19 of 20 cases and cold agglutinins in 17 of the 20 cases However in three adult patients with longlasting excretion CF and TRI antibodies of 7S class exclusively were demonstrated throughout the course of illness (Table 1) Among the 4 cases with the longest persistence of *M pneumoniae* (2½ 4 months) 2 cases had CF and TRI antibodies of 7S class only already 10–20 days after clinical onset one case had some 19S CF and TRI antibodies the 19S antibodies disappearing while the micro-organism still persisted in the throat and the 4th case had a longlasting (4 months) predominance of 19S antibodies

Antibody response in relation to age During studies of the antibody response in family members infected with *M pneumoniae* it was observed that the ratio of 19S to 7S antibodies at any given time period after onset of illness was higher in the children than in their parents Fig 1 illustrates the distribution of antibodies within 19S and 7S immunoglobulins in a 12 year old child and her 43 year old mother In the serum collected from the child 12 days after onset of illness more than 80 per cent of the CF and TRI antibodies were present in the 19S fractions (no 1–4) 5 months after clinical onset antibodies of 7S class predominated The mother,

a red formazan This usually occurred after 5 days The highest serum dilution which prevented a colour change was taken as the titre of inhibiting antibodies The titration of two reference convalescent sera were included in each test

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No excretion or less than 8 days	10	1*	4§	5		1	6	1	3	[illegible]	3	[illegible]
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RESU

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TABLE 2 *Geometric Mean (GM) of Maximum Antibody Titres to* M pneumoniae *and Occurrence of Cold Agglutinins in Relation to Age in 78 Patients with* M pneumoniae *Pneumonia*

Age group years	No of cases	GM of antibody titres CF	TRI	No of cases with pos IHA*	GM of IHA titres in pos cases	No of cases with pos CA
5–19	26	263	88	16/16	449	26/26
20–39	26	218	191	14/16	312	24/26
≧40	26	142	334	11/15	290	15/26

* all cases examined were positive by CF and TRI

per cent) out of 22 patients had an early preponderance of IgM antibodies

Young patients (5–19 years old) usually developed higher titres of CF antibodies than of TRI antibodies whereas patients above 40 years often had higher levels of TRI antibodies as shown in Fig 2 and Table 2 The titres plotted in Fig 2 are the maximum antibody titres observed during a follow up period of at least 4 weeks after clinical onset It appears that higher CF than TRI antibody titres occurred in 23 out of 26 cases of pneumonia in the age group 5–19 years but in only 9 out of 26 pneumonia patients older than 40 years IHA antibodies were demonstrated in all of 16 patients in the age group 5 19 years whereas these antibodies were lacking in 4 out of 15 patients above 40 years who had formed CF and TRI antibodies (Table 2) Cold agglutinins were present in all of 26 young patients (5–19 years) but in only 15 out of 26 patients older than 40 years (Table 2)

Occurrene of antibodies to M pneumoniae

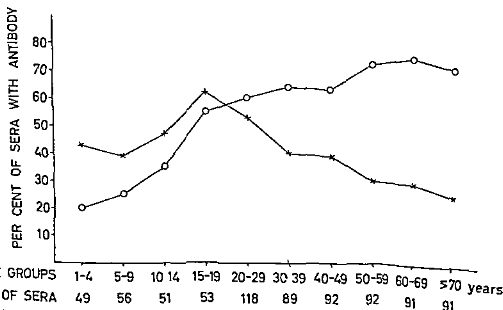


Fig 3 Antibody to *M pneumoniae* in sera from 782 persons of different ages without respiratory illness as measured by CF and TRI ×——× CF antibody in titre ≧1/2 ○——○ TRI antibody in titres ≧1/2

Fig 1 Distribution of CF (×——×) and TRI (□——□) antibodies to *M pneumoniae* in density gradient centrifugation fractions of sera from a 12 year old child and her 43 year old mother Both were cases of pneumonia The fractions were collected from the bottom of the centrifuge tube

however, had CF antibodies of 7S class mainly and TRI antibodies of 7S class exclusively 2 weeks as well as 7 months after onset of illness

Altogether 21 infected cases distributed on 7 families were examined In all these families the children had a higher percentage of IgM antibodies than the parents when compared at approximately the same time after onset of illness

Comparison of the IgM and IgG CF antibody response to *M pneumoniae* in 88 patients of different ages with pneumonia showed that in 17 (77 per cent) out of 22 cases aged 5 19 years IgM antibodies predominated 10–25 days after onset of illness, whereas among adults above 40 years only 5 (23 per cent) out of 22 cases possessed more IgM than IgG CF antibodies at the corresponding time after clinical onset In the intermediate age group (20–39 years) 10 (45

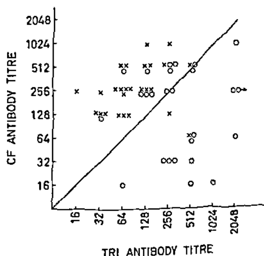


Fig 2 Comparison of maximum CF and TRI antibody titres in 52 patients with *M pneumoniae* pneumonia belonging to two different age groups × – cases 5 19 years old 0 = cases ≧40 years old (O→ TRI titre 1/4096)

TABLE 2 *Geometric Mean (GM) of Maximum Antibody Titres to M pneumoniae and Occurrence of Cold Agglutinins in Relation to Age in 78 Patients with M pneumoniae Pneumonia*

Age group years	No of cases	GM of antibody titres CF	TRI	No of cases with pos IHA*	GM of IHA titres in pos cases	No of cases with pos CA
5–19	26	263	88	16/16	449	26/26
20 39	26	218	191	14/16	312	24/26
>40	26	142	334	11/15	290	15/26

* all cases examined were positive by CF and TRI

per cent) out of 22 patients had an early preponderance of IgM antibodies

Young patients (5–19 years old) usually developed higher titres of CF antibodies than of TRI antibodies whereas patients above 40 years often had higher levels of TRI antibodies as shown in Fig 2 and Table 2 The titres plotted in Fig 2 are the maximum antibody titres observed during a follow up period of at least 4 weeks after clinical onset It appears that higher CF than TRI antibody titres occurred in 23 out of 26 cases of pneumonia in the age group 5–19 years but in only 9 out of 26 pneumonia patients older than 40 years IHA antibodies were demonstrated in all of 16 patients in the age group 5–19 years whereas these antibodies were lacking in 4 out of 15 patients above 40 years who had formed CF and TRI antibodies (Table 2) Cold agglutinins were present in all of 26 young patients (5–19 years) but in only 15 out of 26 patients older than 40 years (Table 2)

Occurrence of antibodies to M pneumoniae

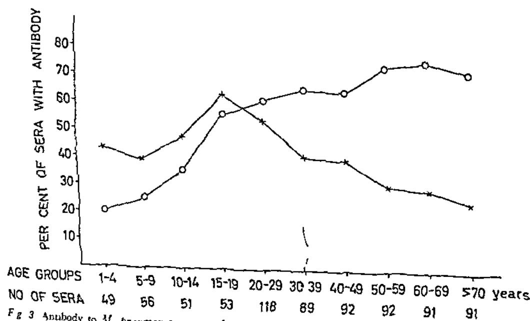


Fig 3 Antibody to M pneumoniae in sera from 782 persons of different ages without resp ... as measured by CF and TRI ×——× CF antibody in titre ≧1/2 ○——○ TRI antibody >1/2

TABLE 3 *Titres of CF and TRI Antibodies to M pneumoniae in 41 Sera Obtained Two to Four Years after M pneumoniae Pneumonia in Relation to Titres in Convalescent Phase Serum*

Antibody titre in convalescent serum	CF antibody titre two to four years after pneumonia							TRI antibody titre two to four years after pneumonia							
	<4	4	8	16	32	64	128	<2	2	4	8	16	32	64	128
1024			1		1		1					2	2		1
512			1		5	3							2	2	1
256		1	2	7	1	1					1	1		2	
128			3	1	3				1	2	4	6	1	1	
64	1		1					2	2		2	2			
32	1	2	3	1							3	1			
16	1														
Total	3	3	11	9	10	4	1	2	3	2	10	12	5	5	2

in relation to age in individuals without respiratory illness Altogether 782 sera from persons of different ages without respiratory illness were examined for CF and TRI antibodies It appears from Fig 3 that the proportion of sera containing CF antibodies (in titre > 1/2) was highest in the age groups 15–19 years (62 per cent) and 20–29 years (53 per cent) and then gradually decreased with increasing age while the proportion of sera which contained TRI antibodies (in titre > 1/2) increased with age and was 70–74 per cent in subjects above 50 years Thus in the age groups below 20 years CF antibodies were more frequent than TRI antibodies whereas in the higher age groups the pattern was reversed

Persistence of antibodies Sera were collected from 41 persons two to four years after they had had *M pneumoniae* pneumonia and the sera were examined for CF and TRI antibodies Table 3 shows the antibody titres of these sera in relation to the titres of the convalescent phase sera Most sera obtained after 2–4 years contained antibodies in titre 1/8 to 1/32 and some sera from cases with high antibody titres during convalescence had titres of 1/64 or 1/128 Only 3 sera lacked detectable CF antibodies (titre <1/4) and 2 sera TRI antibodies (titre <1/2) Examples of the temporal changes of antibody titres as measured by different serological tests during a follow up period of 1–2 years after infection are found in Tables 4 and 5

The 41 sera collected 2–4 years after infection were examined also by IFL for *M pneumoniae* antibodies of IgG class All sera gave a positive reaction when tested in dilution 1/8 In addition, 20 of these sera which had been collected 4 years after infection were tested in dilution 1/8 by IFL with a conjugated anti IgM serum 11 out of 20 sera had demonstrable IgM antibodies Following fractionation of 18 of the 20 above mentioned sera (2 sera were negative in the CF test) by density gradient centrifugation CF antibodies of IgG class were demonstrated in all sera and CF antibodies of IgM class in 10 of the 11 sera which were positive for IgM antibodies by IFL

17 of the sera collected 4 years after infection and shown to contain CF antibodies were examined simultaneously in the CF test against a lipid *M pneumoniae* antigen obtained from Dr *G Kenny*, Seattle, USA and a lipid antigen prepared in this laboratory All 17 sera reacted with both antigens (1/4) but in 7 sera the antibody titre was twofold lower against the antigen from Dr *Kenny*

IgM levels in relation to the 19S (IgM) and 7S antibody responses The serum IgM levels were followed in 33 cases during and after infection with *M pneumoniae* In 25

TABLE 4 *Temporal Change of Serum IgM Levels and of Antibody Titres in 5 Patients with* M *pneumoniae Pneumonia Who Formed Much IgM Antibody*

Case No and age	Time after onset of illness	IgM level in % of standard	CF antibody titre	CF antibody % 19S	TRI antibody titre	TRI antibody % 19S	IHA antibody titre	IHA antibody % 19S	CA titre	Throat culture
	7 d	62	16	nt	<1		<4		16	+
84	24 d	230	256	72	64	30	1024	100	128	+
	62 d	80	128	nt	32	nt	256	nt	32	—
10 yr	4 m	62	64	33	16	nt	64	nt	8	
	24 m	nt	16	nt	8	nt	nt		nt	
	9 d	88	32	nt	8*	nt	256	nt	256	+
129	16 d	145	256	48	64*	nt	2048	nt	512	—
	28 d	125	256	nt	64	nt	1024	nt	256	+
14 yr	95 d	96	256	17	32	nt	512	nt	32	—
	12 m	90	128	nt	16	nt	128	nt	16	
	24 m	nt	64	nt	16	nt	nt		nt	
	8 d	110	<4		<1*		8	nt	8	+
	15 d	160	512	96	128*	83	512	nt	256	+
65	28 d	138	512	95	128	83	512	nt	16	+
	44 d	128	256	82	128	55	256	nt	8	+
19 yr	3 m	106	128	82	64	57	64	nt	8	—
	5 m	102	64	93	16	nt	32	nt	<8	
	26 m	nt	16	40	4	nt	nt		nt	
	9 d	132	16	nt	8	nt	32		32	+
5	24 d	284	2048	20	256	8	4096	94	256	+
	46 d	228	2048	23	256	11	4096	nt	64	+
32 yr	81 d	200	1024	24	256	12	4096	nt	32	—
	6 m	164	1024	19	128	0†	1024	nt	8	
	12 m	144	512	24	128	0	512	85	nt	
	11 d	198	64	76	32*	nt	128	nt	128	
6	20 d	300	512	76	512	73	1024	nt	128	+
	32 d	318	256	82	512	81	1024	nt	256	—
42 yr	4 m	198	128	71	128	26	256	nt	32	—
	20 m	nt	64	nt	32	nt	nt		nt	

* sera collected during treatment of patient with tetracyclines
† 0 = 100 per cent 7S
nt not tested
titre – reciprocal of serum dilution
d days m = months

of these patients the IgM concentration increased by more than 30 per cent and in 12 patients by more than 100 *per cent* The change in IgM levels in patients which had formed considerable amounts of *M pneumoniae* antibodies of IgM class was compared with the serum IgM content in cases who had *responded with 7S* antibodies almost exclusively The titres and immunoglobulin classes of *M pneumoniae* antibodies in 5 and 6 cases, respectively, from these two groups are presented in Tables 4 and 5 In several cases the immunoglobulin class of the IHA antibodies was not determined, but it has been shown previously that these antibodies belong to the IgM class exclusively *or* predominantly at least during the firs after onset of illness (3) Table 4

TABLE 5 *Serum IgM Levels and Temporal Change of Antibody Titres in 6 Patients Who Responded to M* pneumoniae *Infection with antibodies of 7S Class almost Exclusively 5 Cases Had Pneumonia and One Case (no 141) Bronchitis with Fever*

Case No and age	Time after onset of illness	IgM level in % of standard	CF antibody titre	CF antibody % 19S	TRI antibody titre	TRI antibody % 19S	IHA antibody titre	IHA antibody % 19S	CA titre	Throat culture
398	8 d	44	<4		8	nt	<4		<8	+
	18 d	52	64	0†	1024*	0	<4		<8	+
67 yr	39 d	44	64	nt	2048	nt	<4		<8	—
	60 d	42	64	0	2048	0				
	6 d	110	4		2*		<4		16	+
77	13 d	104	32	0	128*	0	8	nt	16	—
	24 d	104	32	nt	256	nt	4	nt	16	—
49 yr	51 d	100	32	0	128	0	<4		16	—
	5 m	100	16	0	64	0	<4			
	26 m	nt	8		64					
	6 d	84	<4		<1*		<4		<8	+
	13 d	85	64	0	256*	0	<4		<8	—
2	21 d	90	64	nt	512	nt	<4		<8	—
	58 d	81	32	0	256	0	<4		<8	—
53 yr	86 d	89	32	0	128	0				—
	12 m	82	8	nt	64	nt				
	28 m	nt	4		32					
	3 d	79	4		<1*		<4		<8	+
93	10 d	67	16	0	128*	0	32	71	<8	—
	27 d	75	32	nt	128	nt	16	nt	<8	—
34 yr	4 m	79	16	0	32	0	8	nt	<8	—
	23 m	nt	8	nt	16	nt	nt			
	0 d§	140	<4		8	nt	<4		<8	—
133	10 d	172	32	0	256*	0	<4		<8	—
	29 d	135	16	nt	128	nt	<4		8	—
45 yr	5 m	135	16	0	64	0	<4		<8	
	24 m	nt	4	nt	64	nt				
	2 d	96	<4		<1*		<4		<8	+
141	8 d	nt	8	0	64*	nt	<4		<8	—
	23 d	100	16	0	128	0	64	100	<8	+
39 yr	4 m	104	16	nt	64	nt	16	nt	<8	+
	24 m	nt	4	nt	32	nt	nt			

§ 2 days before clinical onset
* Sera collected during treatment with tetracyclines
† 0 = 100 per cent 7S
nt – not tested
titre = reciprocal of serum dilution
d – days m = months

temporal changes of the IgM level in relation to time after onset of illness in 5 patients with high titres of IgM antibodies and of cold agglutinins The IgM content increased during the first few weeks after onset of illness and then fell off gradually Among patients with IgM antibody responses of approximately the same magnitude some cases showed a much greater elevation of IgM than other cases (for instance case 84 com

TABLE 6 *Effect of Absorption of* M pneumoniae *Antibodies and Cold Agglutinins on the Serum IgM Level in 8 Cases of* M pneumoniae *Infection*

Case No	Time after onset of illness	Absorbed with	Antibody titre IHA[1] or CF	Antibody titre CA	IgM level in % of standard	IgM level in % of standard Corrected for dilution during abs	% reduction of total IgM*	Reduction of IgM in % of IgM increase during inf
20	42 d	—	224[1]	448	270			
		M pn	<7[1]	nt	189	211	22	36
		RBC	nt	<7	238	270	0	0
		RBC & M pn	<7[1]	<7	175	216	20	33
	14 m	—	32[1]	8	104			
138	9 d	—	5[1]	20	118			
	13 d	—	192[1]	384	183			
		M pn	<6[1]	nt	141	146	20	57
		RBC	nt	<6	147	182	0 5	1 5
		RBC & M pn	<6[1]	<6	120	155	15	43
415	4 d	—	<4	<8	100			
	11 d	—	192	192	243			
		M pn	<6	nt	177	194	20	34
		RBC	nt	<12	213	230	5	9
		RBC & M pn	<6	<12	135	168	31	52
563	10 d	—	16	128	80			
	25 d	—	80	1280	165			
		M pn	<5	nt	138	151	8	16
		RBC	nt	<10	130	147	11	21
		RBC & M pn	<5	<10	113	138	16	32
565	6 d	—	8	16	180			
	27 d	—	224	448	350			
		M pn	7	nt	282	307	12	25
		RBC	nt	<7	285	341	2 6	5
		RBC & M pn	7	<7	217	288	18	36
266	7 d	—	<4	<8	80			
	21 d	—	224	224	525			
		M pn	<7	112	329	363	31	36
		RBC	224	<14	420	521	0 8	0 9
379	9 d	—	32	256	125			
	31 d	—	512	512	312			
		M pn	<8	256	172	205	34	57
		RBC	256	<16	248	314	0	0
515	7 d	—	8	<8	56			
	29 d	—	256	1024	134			
		M pn	<4	nt	106	111	17	29
		RBC	256	<8	116	133	0,7	1,3

RBC = red blood cells
d = days m = months
* after correction for dilution during absorption

seroconversion *Cordero et al* (12) observed that among individuals with clinically overt illness 10 per cent had lost demonstrable TRI antibody after 2 years whereas the corresponding figure was 71 per cent for subjects whose infection was asymptomatic A similar trend was noted also for CF antibodies In the present study as well as in a study by *Nakamura et al* (29) all the individuals examined for persisting antibodies had pneumonia during *M pneumoniae* infection *Nakamura et al* demonstrated CF antibodies (in titre $\geq 1/8$) in 9 out of 14 subjects and MI antibody (titre $\geq 1/2$) in 10 out of 14 subjects 2–4 years after infection

The distribution of CF and TRI antibodies in subjects of different ages without respiratory illness showing a higher incidence of CF than TRI antibodies in children and young adults and a higher incidence of TRI than CF antibodies in older people was similar to the antibody pattern recently demonstrated in the healthy population in Czechoslovakia (33) These results are in line with the observation that, following infection young patients usually developed higher titres of CF than of TRI antibodies whereas in adults over 40 years it was the reverse In the Czech study of healthy people the percentage of sera with CF antibody (in titre >8) was higher and the percentage of sera positive for MI (TRI) antibody in middleaged and old people was lower than in the present investigation In similar investigations performed in the USA (17 38) the incidence of CF and TRI antibodies in the general population has been lower than that found in the Swedish population This may indicate that *M pneumoniae* infection has been more frequent in Sweden but the differences may also be due to technical differences in performance of the serological tests

It has been shown previously that serum IgM levels rise during infection with *M pneumoniae* whereas the IgG and IgA levels vary only slightly and usually remain within the normal range (15 30 31) Most patients examined in the present study showed an increase of IgM but in those patient who developed *M pneumoniae* antibodies almost exclusively of 7S class the IgM level changed only slightly

Absorption of the *M pneumoniae* antibodies in sera with elevated IgM resulted in a 8–34 per cent reduction of the total IgM whereas absorption of cold agglutinins had little effect on the IgM level in 7 out of 8 sera examined It should be noted that the error involved in the absorption method did not allow the demonstration of a small reduction (<7 per cent) of IgM However in one case the cold agglutinins accounted for 11 per cent of the total serum IgM *Fei (15)* reported partial lowering of the IgM level after absorption of cold agglutinins in one case of cold agglutinin syndrome after *M pneumoniae* infection *Wollheim et al* (42) found that absorption of the polyclonal cold agglutinins occurring in patients with pneumonia redued the IgM level by 0–24 per cent while removal of monoclonal cold agglutinins from sera with M components lowered the total IgM by as much as 75–95 per cent

In the 8 sera examined by absorption in the present work absorbable *M pneumoniae* antibody and cold agglutinins accounted for 30–57 per cent of the IgM increase which could be demonstrated during the infection *Streptococcus MG* agglutinins and tissue antibodies of IgM class which appear in a proportion of patients with *M pneumoniae* infection were probably not responsible for any additional amount of IgM since these antibodies also are absorbed with *M pneumoniae* antigen (5 25) One conceivable explanation of the remaining IgM elevation might be the existence of *M pneumoniae* antibodies of IgM class of low avidity that were not adsorbed under the conditions used However in some cases the acute phase serum contained *M pneumoniae* antibodies in higher titre than the absorbed convalescent phase serum and still the IgM level was significantly higher in the latter serum A large part of the IgM increase associated with *M pneumoniae* infection would thus seem to be attributable to so called "non specific immunoglobulin production Those

patients who developed little or no demonstrable *M pneumoniae* antibodies of IgM class also seemed to produce insignificant amounts of nonspecific IgM as judged by the fairly stationary serum IgM levels in these cases Elevated IgM concentrations occur in infections other than those caused by *M pneumoniae* as for example trypanosomiasis (28), malaria (40), infectious mononucleosis (41), acute hepatitis (43) and syphilis (22) but it is not yet clear to what extent the IgM elevation represents a specific antibody response to the agent responsible for the infection Elevations of IgM also have been demonstrated in sera from children immunized with typhoid vaccine (1) Absportion assays showed that typhoid antibody acounted for 18 per cent to 32 per cent of the total IgM in these immune sera, corresponding to most of the IgM increase that had occurred after immunization However, there is evidence from other studies that injection with bacterial lipopolysaccharides (endotoxins) may elicit nonspecific stimulation of immunoglobulin and natural antibody production (see 8) Approximately half of the amount of IgG produced by animals in response to immunization with various antigens has been attributed to nonspecific immunoglobulins (see 8) Nonspecific immunoglobulin production has recently been discussed in detail by *Burnet* (8)

I wish to thank Dr *Renee Norberg* for helpful advice regarding the radial immunodiffusion method

The skilled technical assistance of *Lana Ronnerfors* and *Helen Linder* is gratefully acknowledged

This work was supported by the *Swedish Medical Research Council* (Project No B71 16X-2380-04)

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RED CELLS, ERYTHROAGGLUTINATING ACTIVITY OF PHYTOHAEMAGGLUTININ, AND LYMPHOCYTE STIMULATION

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Lymphocytes purified from human blood responded poorly to each of two different fractions of a phytohaemagglutinin preparation. One of the fractions agglutinated red cells and a mixture of [illegible] leucocytes and platelets (leucocytes-platelets); the lymphocyte response to this fraction was increased by red cells as well as by leucocytes-platelets. The other phytohaemagglutinin fraction agglutinated leucocytes-platelets but not red cells; the response to this fraction was increased by leucocytes-platelets but not by red cells. The results are compatible with the assumption that a component of phytohaemagglutinin bound to the red cell membrane induces lymphocyte stimulation more effectively than does [illegible] lymphocytes.

In [illegible] (Tarnvik 1971) [illegible] RBC [illegible] RBC.

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MATERIALS AND METHODS

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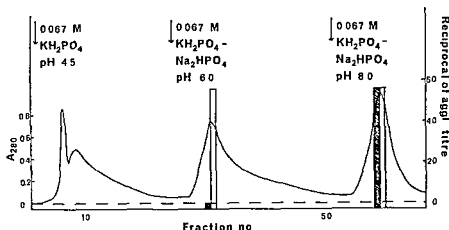


Fig 1 SP Sephadex C 50 chromatography of 50 mg Bacto—PHA P (Difco) The solid line indicates the absorbancy at 280 nm of eluted material The open bars indicate leucocyte platelet agglutinating, and the black bars erythroagglutinating activities of the pooled fractions of the peaks Agglutinating activity is expressed as the reciprocal of the highest agglutinating dilution of a solution containing 320 µg protein per ml

H PHAP was studied The RBC were either agglutinated by isoantiserum or nonagglutinated The lymphocytes and the RBC membranes were obtained from the same blood sample Fifty µl of L PHAP or H PHAP were added to each lymphocyte culture to a final protein concentration of 10 µg per ml Then 0 3 ml of the suspension of agglutinated or nonagglutinated RBC membranes was added per lymphocyte culture

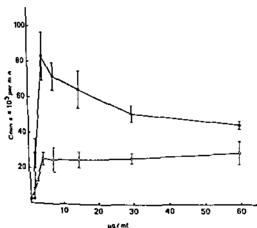


Fig 2 The lymphocyte response to various concentrations of L PHAP (○——○) and H PHAP (●——●) The lymphocyte preparation contained 30 RBC and 2 non lymphocytic leucocytes per 100 lymphocytes The incorporation of ^{3}H thymidine into DNA was measured The means and standard deviations of 5 cultures are indicated

RESULTS

Lymphocytes were incubated in the presence of the chromatographed material L-PHAP agglutinated leucocytes-platelets but did not agglutinate RBC, whereas *H-PHAP* agglutinated both these cell preparations (Fig 1) The incorporation of ^{3}H thymidine into DNA induced by L-PHAP was increased only slightly or not at all by addition of RBC, but was greatly increased by addition of leucocytes platelets (Table 1) The incorporation induced by H PHAP was greatly increased by the addition of RBC as well as by the addition of leucocytes platelets

The incorporation of ^{3}H-thymidine induced by H-PHAP was increased with the density of RBC membranes in the culture RBC membranes did not increase the incorporation induced by L-PHAP (Fig 3) The incorporation induced by L PHAP was not increased by RBC membranes, even if the RBC membranes were agglutinated by blood group specific isoantibodies (Fig 3)

DISCUSSION

The lymphocyte response to L PHAP was enhanced by leucocytes platelets but not by RBC, whereas the response to H-PHAP was

Culture medium The culture medium consisted of 80 per cent Parker TCM 199 (Flow Laboratories, Irvine Ayrshire, Scotland) and 20 per cent pooled inactivated (56° C, 30 min) human serum The culture medium also contained 150 units of benzylpenicillin (KABI AB, Stockholm, Sweden) and 150 µg of streptomycin sulphate (Glaxo Laboratories Ltd, Greenford, England) per ml

Phytohaemagglutinin Bacto-phytohaemagglutinin P (PHAP, Difco Laboratories, Detroit, Michigan) was chromatographed as described by *Weber et al* (1967) SP Sephadex C 50 (Pharmacia, Uppsala, Sweden) was allowed to swell in 0 5 N KCl overnight It was then washed in 0 067 M KH_2PO_4 at pH 4 5 and packed at +4° C in a 14×240 mm column

Fifty mg of PHAP were applied to the column, which was then washed with the pH 4 5 buffer Elution was performed in two steps with 0 067 M KH_2PO_4-Na_2HPO_4 at pH 6 0 and at pH 8 0 (Fig 1) The fractions in the pH 6 0 peak were collected into one pool and those in the pH 8 0 peak into another The pools were dialyzed against physiological saline at +4° C and frozen at —20° C Their protein content was determined according to *Lowry et al* (1951) using weighed amounts of PHAP as standard These two pools were referred to as *L PHAP* and *H PHAP* They had agglutinating properties similar to L-PHAP and H PHAP described by *Allen et al* (1969), although it cannot be claimed that they were identical to the latter

The fractions in the peak obtained at pH 4 5 were not studied They have been found to lack agglutinating and lymphocyte stimulating activities (*Weber et al* 1967)

Preparation of RBC and RBC membranes RBC were obtained from the same sample as used for preparation of lymphocytes Blood was filtered through a nylon fibre column and centrifuged Plasma was discarded and the RBC were suspended to appropriate densities in culture medium RBC membranes were prepared by hypotonic treatment as previously described (*Tarnvik* 1971)

Preparation of leucocytes platelets Buffy coat of non filtered blood from 100 ml of the same sample as used for preparation of lymphocytes was centrifuged in colloidal silica polyvinylpyrrolidone (*Pertoft et al* 1968 *Tarnvik* 1970 a) and cells from the mononuclear band were washed and diluted in culture medium This preparation (leucocytes platelets) consisted of monocytes lymphocytes and platelets in about the same relative proportions as in whole blood, but contained very few RBC and polymorphonuclear leucocytes The preparation was treated with mitomycin C (Sigma Co, St Louis, Missouri) in order to prevent DNA synthesis (*Bach & Voynow* 1966) The cells were suspended to appropriate densities in culture medium These

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microscopically

Lymphocyte cultures The lymphocytes were pended *in culture medium to* 0.7×10^6 cells per ml The suspension was distributed in 1 5 ml aliquots in test tubes The tubes were loosely capped with aluminium foil and incubated for 62 hours at 37° C in humidified air supplemented with 5 per cent (v/v) CO Fourteen hours before the end of the culture period, 1 5 µCi of [methyl ³H] thymidine (specific activity 6 7 Ci per mmole, New England Nuclear Corp, Boston Massachusetts) in 30 µl of deionized water was added to each tube The radioactivity of ³H thymidine incorporated into DNA was measured (*Borjeson et al* 1966 *Tarnvik* 1970 b)

The effect of RBC and leucocytes platelets on the lymphocyte response to L-PHAP and H PHAP was studied To each culture, 50 µl of L PHAP or H-PHAP were added to a final protein concentration of 10 µg per ml of culture *At this concen*tration the incorporation of ³H thymidine into DNA was about optimal for both L-PHAP and H PHAP (Fig 2) To each culture 0 2 ml of an RBC or leucocyte platelet suspension was added

The effect of various densities of *RBC mem*branes on the lymphocyte response to L PHAP and

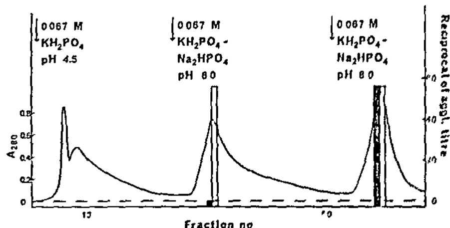


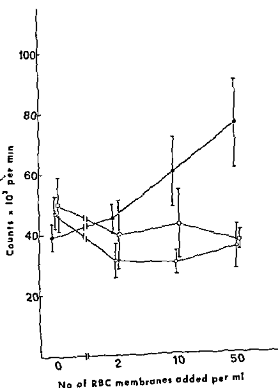
Fig 1 SP-Sephadex C-50 chromatography of 50 mg Bacto-PHA P (Difco). The solid line indicates the absorbance at 280 nm of eluted material. The open bars indicate leucocyte/platelet agglutinating and the black bars erythroagglutinating activities of the pooled fractions of the peaks. Agglutinating activity is expressed as the reciprocal of the highest agglutinating dilution of a solution containing [illegible] μg protein per ml.

TABLE I *Effect of RBC and Leucocytes-Platelets on the Lymphocyte Response to L-PHAP and H-PHAP*

Exp no	Cell preparation added	No of cells added per ml of culture	L PHAP	H-PHAP	NaCl
1	RBC	10^7 RBC	4,373±978*	30,531±8,485	
	RBC	2 5×10^6 RBC	5,279±1,165	23,959±2,409	
	Leucocytes platelets	4 ×10^6 leucocytes	14,022±2,599	18,887±5,276	
	Leucocytes platelets	10^6 leucocytes	9,960±1,863	13,969±2,599	
	No cells		4,594±672	6,820±2,596	18±5
2	RBC	10^7 RBC	448±195	6,086±1,671	
	RBC	2 5×10^6 RBC	190±119	6,260±505	
	Leucocytes platelets	2 ×10^6 leucocytes	845± 95	317± 72	
	Leucocytes platelets	0 5×10^6 leucocytes	235± 85	141± 19	
	No cells	–	86± 23	82± 12	21±3
3	RBC	5 ×10^8 RBC	18,482±3,462	74,061±8,632	
	Leucocytes platelets	10^6 leucocytes	38,951±1,802	56,763±1,473	
	No cells	–	16,763±1,473	17,747±5,970	61±12

* Mean counts per min ± S D of 4 or 5 cultures

L-PHAP and H-PHAP were added to lymphocyte cultures to a final concentration of 10 µg of protein per ml of culture, and the incorporation of ³H thymidine into DNA was measured A preparation consisting of mononuclear leucocytes and platelets (leucocytes platelets) was treated with mitomycin C and added to some cultures, to other cultures RBC were added Both of the added preparations were obtained from the same blood sample as the lymphocytes



enhanced by leucocytes-platelets as well as by RBC It has been suggested that a complex of phytohaemagglutinin and RBC membrane induces lymphocyte stimulation more effectively than does phytohaemagglutinin free in solution (*Tärnvik* 1971) The present results confirm the assumption that binding of phytohaemagglutinin to the RBC membrane is favourable for the induction of stimulation

Fig 3 Effect of varying numbers of RBC membranes on the lymphocyte response to L-PHAP and H PHAP The concentration of L-PHAP or H PHAP was 10 µg per ml of culture Various numbers of RBC membranes were suspended in serum, containing isoagglutinins directed against the RBC membranes This RBC suspension was added to lymphocyte cultures The means and standard deviations of 4 cultures are indicated

□——□ L PHAP, isoagglutinins present
○——○ L PHAP, isoagglutinins absent
●——● H-PHAP, isoagglutinins absent

The agglutinating and stimulating activities of L-PHAP (*Rivera & Mueller* 1966, *Weber* 1969, *Börjeson* 1970) as well as of H PHAP (*Rigas & Johnson* 1964, *Rigas & Head* 1969, *Börjeson* 1970) are probably due to different parts of the same molecule However, the possible relationship between the agglutinating and lymphocyte stimulating activities has not been clarified *Allen et al* (1969) and *Johnson & Kirkpatrick* (1970) have reported that the lymphocyte response to H PHAP is facilitated by the presence of RBC

The present results are compatible with the indication that the enhancing effect of RBC is related to the binding of phytohaemagglutinin to the RBC membrane It is possible that the effect of the leucocyte platelet preparation is due to an analogous phenomenon These interpretations are compatible with the view that agglutination and lymphocyte stimulation are caused by different parts of the same molecule

The results are incompatible with the possibility that the RBC serve a nutritive function in lymphocyte stimulation by phytohaemagglutinin If this were the case, RBC membranes would be expected to increase the response to L-PHAP as well

RBC membranes did not increase the lymphocyte response to L-PHAP, even when the RBC membranes were present in agglutinated state This contradicts the possibility that the effect of RBC membranes in phytohaemagglutinin culture is due to the creation of a favourable microenvironment depending on their agglutinated state

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Grants for this investigation have been received from the *Faculty of Medicine University of Umeå* and from the *Helge and Manhild Johansson foundation* I am grateful to Dr *Bertil Cedergren*, who kindly provided blood samples

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in *Escherichia coli* form the subject of the present report

METHODS

Bacteriological Routine Tests

Media and tests, unless specifically mentioned below, were as described by *Kaufmann* (6)

KCN test and *decarboxylase tests* according to *Møller* (12, 13)

The ONPG test according to *Bulow* (2)

The oxidase test according to *Kovacs* (7)

The urease test was made by preparing a heavy suspension of bacteria from an agar plate in a solution containing urea 1 per cent, KH_2PO_4 0 1 per cent, K_2HPO_4 0 1 per cent, NaCl 0 5 per cent, phenol red (1 500) 5 ml/litre, pH 7 1 Reading after 2, 4 and 20 hours

The phenylalaninedeaminase test was made by preparing a heavy suspension of bacteria from an agar plate in 1 ml of a watery, non buffered solution of 0 2 per cent L phenylalanine The reagent (*13 5 g $FeCl_3$, 6H O, 5 ml N HCl, 100 ml distilled* water) was added after 3 hours and a very dark green colour is required to rate the test as positive

Routine test for H_2S production The test used in routine examination is stab inoculation in a tubed ferrous chloride gelatine medium originally described by *Kristensen et al* (8) and since then used regularly in this country The tubes are incubated at 22°C Readings for H_2S production are made after 2 and 4 days A heavy black precipitate is required to rate the test as definitely positive Less intense blackening is recorded as weakly positive Unfortunately different batches of the medium may vary considerably in the degree of blackening that is produced by the same strain

New Special Tests for H_2S Production

Demonstration of hydrogensulphide production in individual colonies Formation of hydrogensulphide is demonstrated in individual surface colonies by a centrally located black colour after 24 hours aerobic incubation on the following medium human placenta broth (*Kauffmann* (6)), sodium thiosulphate pentahydrous 8 5 g/litre ferric citrate 1 g/litre, Danish agar 25 g/litre (approx), pH 7 6

Demonstration of hydrogensulphide production from media containing different sulphur compounds A series of defined H S indicating growth media each containing one of five sulphur compounds was prepared with the following final composition per litre 10 ml of a double strength concentrated mineral base as described by *Cohen Bazire, Sistrom & Stanier* (3) with exclusion of thiamine, biotin and niacin, 8 ml 10 per cent ammonium chloride, 100 ml 0 4 M phosphate 0 2 M citrate acid buffer, pH 7 2, 3 ml of a 30 per cent D galactose* solution as a source of carbon energy and reduction, 5 ml of a freshly prepared 10 per cent solution of ferrous chloride as detector of H S formation, 1 0 g oxoid ion agar No 2, and the various sulphur sources in the following equimolar sulphur amounts (except for Na_2SO_3) 2 4 g Na_2SO_4 anhydrous, 1 0 g Na_2SO_3 anhydrous 1 3 g $K_2S_4O_6$ anhydrous, 2 1 g $Na_2S_2O_3$,$5H_2O$, 3 0 g L cysteine hydrochloride $1H_2O$ The medium was dispensed in a 10 cm high column in 10 × 155 mm glass tubes with paraffin treated cork stoppers As a control for *growth and H_2S formation from* minor amounts of sulphate in the concentrated base a medium without added sulphur source was used

Except for the cysteine medium the keeping quality at room temperature was good for several months The cysteine medium tends to show spontaneous blackening initiated by oxidative conversion of cysteine, but this can be prevented by an overlayer of paraffin oil

By means of a straight wire the media were inoculated to the bottom of the tubes from an overnight plate culture The reading was performed after 1, 2, 3, 4 and 7 days of incubation at 35°C

Test for Sensitivity to Antibiotics

The tests were performed by a prediffusion disc technique described in detail by *Thomsen* (16) By this method a gram negative rod is rated as penicillin resistant when inhibited by a 6 mm disc containing 100 units of sodium benzylpenicillinate

Serological Methods

Serotyping of the strains and antiserum production were performed according to standard procedures (6) All strains were tested in 150 different O and 50 different H antisera

Methods in the Genetic Transfer Experiments

A 500 ml Erlenmeyer flask *containing 60 ml* fresh broth was inoculated with 1 0 ml of overnight broth culture of the donor and 0 1 ml of the recipient culture and incubated at *37°C for 24* hours 0 1 ml aliquots of dilutions 10^{-3}, 10^{-4} and 10^{-5} of the mixture were spread on to the placenta thiosulphate ferric citrate medium containing streptomycin (100 µg/ml) or nalidixic acid (20 µg/ml) to inhibit growth of the donor cells The plates were read after 24 hours incubation at 37°C H_2S production may not be detected if more than a few hundred colonies grow on a plate with a diameter of 8 5 cm Thus the dilution which produced this number of colonies or less, was

* In later experiments D glucose has been used in media containing thiosulphate with equally good or better results

chosen for the final experiment in which not less than 600 but usually about 1000 colonies were inspected H S positive recipient colonies were subsequently purified In the 24 cases where the donor culture was sensitive to streptomycin a streptomycin resistant culture was used as recipient this being either a derivative of the test strain of O antigen 100 (H509a) W3479, requiring histidine and isoleucine or a K12 strain W1607, requiring methionine A mutant of W3479 resistant to nalidixic acid D726 was employed as recipient in experiments involving the two donor strains resistant to streptomycin

Efficiency of transfer was measured as the number of H S producing recipient cells per input donor cell after 30 minutes mixed incubation of equal numbers of refreshed donor and recipient cells

RESULTS

Isolation Distribution and Frequency of the Variants

The first three variants of this kind occurred in May and June 1969 in a series of isolates received for identification from a hospital running a systematic study of urinary tract infections

Once the unusual character of the isolates was realized other microbiological laboratories were asked to look for similar strains and by the end of June 1970 altogether 26 isolates had been collected Only four laboratories used methods which permitted them to detect the variant and from these four laboratories altogether 22 of the variants were received

The observation period for the study is 14 months More isolates have been received after June 1970 but are not included

Data on the origin and distribution of the strains are presented in Table 1

Two isolates Nos 1 and 3 belonging to different fermentation types and serotypes are from one patient In all other cases only one isolate from each patient has been included even when more than one strain were isolated since the additional strains were found to be identical with the original one Two patients were seen in outpatient departments the rest were hospitalized at the time of the isolation of the variant

As far as our information goes there is nothing to suggest that the H S producing variants differed from ordinary *E coli* in pathogenic significance

As most specimens came from females above 40 years of age having urinary tract infections it is likely that many of the patients were or had recently been subjected to antibiotic treatment

To obtain an estimate of the relative frequency of the H S producing variant among *E coli* isolates information was obtained from the three laboratories having 6 or 7 variants each about the number of ordinary *E coli* isolated in one year This figure varied from 3000 to 6000 so the frequency of H S positive *E coli* may be estimated to be at least between 0 1–0 2 per cent of all *E coli* isolates

Biochemical Characteristics and Antibiotic Sensitivity Pattern

All strains were examined by standard tests on isolation and again when received at the Statens Seruminstitut Furthermore, the set was examined a third time for properties such as acid production from sucrose, dulcitol and salicin in order to establish as firmly as possible the stability of the fermentation types

All strains were gram negative rods without any special morphological characteristics Most of them were motile but 7 strains were non motile They grew well on the ordinary laboratory media producing *E coli* like colonies on agar plates and modified Drigalski agar plates

The following tests were positive in all strains reduction of nitrate to nitrite growth in a mineral medium with ammonium salt as sole nitrogen source and glucose as carbon and energy source, indol production, acid formation from arabinose xylose rhamnose, glucose, lactose and mannitol, gas production from glucose and mannitol, and the ONPG test

The following tests were negative in all strains oxidase reaction, Voges Proskauer test urease test, phenylalaninedeaminase test, gelatine liquefaction tests, growth in Møller's KCN medium, growth in a fluid mineral

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D ... and ... cent ... format... various s... sulphur... Na_2SO_4 an... $K_2S_4O_6$ and... L cysteine h... dispensed in a... glass tubes with... a control for g... minor amounts o... base a medium wi... used

Except for the cy... quality at room tempe... months The cysteine... spontaneous blackening ... version of cysteine, but th... overlayer of paraffin oil

By means of a straight ... inoculated to the bottom of... overnight plate culture The re... after 1, 2, 3, 4 and 7 days of ...

Test for Sensitivity to Antibiotics

The tests were performed by a pr... technique described in detail by *Th*... By this method a gram negative rod... penicillin resistant when inhibited by a ... containing 100 units of sodium benzylpe...

Serological Methods

Serotyping of the strains and antiserum production were performed according to standard procedures (6) All strains were tested in ... different O and 50 different H antisera

Methods in the Genetic Transfer Experiments

A 500 ml Erlenmeyer flask containing 60 ml fresh broth was inoculated with 1 0 ml of overnight broth culture of the donor and 0 1 ml of the recipient culture and incubated at 37°C for 24 hours 0 1 ml aliquots of dilutions 10^{-3} 10^{-4} and 10^{-5} of the mixture were spread on to the placenta thiosulphate ferric citrate medium containing streptomycin (100 μg/ml) or nalidixic acid (20 μg/ml) to inhibit growth of the donor cells The plates were read after 24 hours incubation at 37°C H_2S production may not be detected if more than a few hundred colonies grow on a plate with a diameter of 8 5 cm Thus the dilution which produced this number of colonies or less was

* In later experiments D glucose has been used in media containing thiosulphate with equally good or better results

chosen for the final experiment in which not less than 600, but usually about 1000 colonies, were inspected H_2S positive recipient colonies were subsequently purified In the 24 cases where the donor culture was sensitive to streptomycin a streptomycin resistant culture was used as recipient this being either a derivative of the test strain of O antigen 100 (H509a), W3479, requiring histidine and isoleucine, or a K12 strain W1607, requiring methionine A mutant of W3479 resistant to nalidixic acid D726, was employed as recipient in experiments involving the two donor strains resistant to streptomycin

Efficiency of transfer was measured as the number of H_2S producing recipient cells per input donor cell after 30 minutes mixed incubation of equal numbers of refreshed donor and recipient cells

RESULTS

Isolation, Distribution and Frequency of the Variants

The first three variants of this kind occurred in May and June 1969 in a series of isolates received for identification from a hospital running a systematic study of urinary tract infections

Once the unusual character of the isolates was realized other microbiological laboratories were asked to look for similar strains and by the end of June 1970 altogether 26 isolates had been collected Only four laboratories used methods which permitted them to detect the variant and from these four laboratories altogether 22 of the variants were received

The observation period for the study is 14 months More isolates have been received after June 1970 but are not included

Data on the origin and distribution of the strains are presented in Table 1

Two isolates, Nos 1 and 3 belonging to different fermentation types and serotypes are from one patient In all other cases only one isolate from each patient has been included even when more than one strain were isolated since the additional strains were found to be identical with the original one Two patients were seen in outpatient departments, the rest were hospitalized at the time of the isolation of the variant

As far as our information goes, there is nothing to suggest that the H_2S producing variants differed from ordinary *E coli* in pathogenic significance

As most specimens came from females above 40 years of age having urinary tract infections, it is likely that many of the patients were or had recently been subjected to antibiotic treatment

To obtain an estimate of the relative frequency of the H_2S producing variant among *E coli* isolates information was obtained from the three laboratories having 6 or 7 variants each about the number of ordinary *E coli* isolated in one year This figure varied from 3000 to 6000, so the frequency of H S positive *E coli* may be estimated to be at least between 0 1–0 2 per cent of all *E coli* isolates

Biochemical Characteristics and Antibiotic Sensitivity Pattern

All strains were examined by standard tests on isolation and again when received at the Statens Seruminstitut Furthermore, the set was examined a third time for properties such as acid production from sucrose, dulcitol and salicin in order to establish as firmly as possible the stability of the fermentation types

All strains were gram negative rods without any special morphological characteristics Most of them were motile, but 7 strains were non motile They grew well on the ordinary laboratory media producing *E coli* like colonies on agar plates and modified Drigalski agar plates

The following tests were positive in all strains reduction of nitrate to nitrite, growth in a mineral medium with ammonium salt as sole nitrogen source and glucose as carbon and energy source, indol production, acid formation from arabinose, xylose, rhamnose, glucose, lactose and mannitol, gas production from glucose and mannitol, and the ONPG test

The following tests were negative in all strains oxidase reaction, Voges Proskauer test, urease test, phenylalaninedeaminase test, gelatine liquefaction tests, growth in Møller's KCN medium, growth in a fluid mineral

4 •

TABLE 1 *The H_2S Producing E coli Variants Arranged According to Date of Isolation with Data on Origin*

Variant no	Date of isolation	Patient sex	Patient age	Kind of specimen	Hospital	Area
1	May 1969	♀	37	Urine	Roskilde	A
2	June –	♀	58	Urine	Roskilde	A
3	June –	♀	37	Urine	Roskilde	A
4	July –	♀	54	Urine	Hjørring	B
5	Aug –	♀	82	Blood	Gamles By	A
6	Aug	♀	69	Urine	Alborg S	B
7	Nov –	♀	79	Urine	Alborg N	B
8	Nov –	♀	33	Urine	Rigshospitalet	A
9	Nov –	♀	44	Peritoneal swab	Alborg S	B
10	Dec –	♀	15	Duodenal aspiration	Alborg N	B
11	Jan 1970	♀	67	Anal fistula swab	Frederiksberg	A
12	Jan –	♂	78	Tracheal swab	Rigshospitalet	A
13	Jan –	♀	71	Urine	Århus	C
14	Jan –	♀	81	Urine	Frederiksberg	A
15	Jan –	♀	47	Urine	Århus	C
16	Feb –	♀	19	Urine	Grenå	C
17	Apr –	♀	75	Urine	Frederiksberg	A
18	May	♂	55	Urine	Frederiksberg	A
19	May –	♂	68	Jejunal aspiration	Ålborg N	B
20	May –	♂	Newborn	Umbilical swab	Ålborg N	B
21	May –	♂	68	Urine	Frederiksberg	A
22	June	♂	53	Urine	Frederiksberg	A
23	June	♀	55	Lung tissue	Århus	C
24	June –	♂	66	Pus from retro peritoneal abscess	Århus	C
25	June –	♂	71	Urine	Århus	C
26	June	♂	54	Intraperitoneal dialysis catheter	Århus	C

Main geographical areas A – Copenhagen and surroundings
B = Alborg and surroundings
C – Århus and surroundings

medium with ammonium salt and citrate as sole carbon and energy source, and acid production from inositol and cellobiose Arginine- lysine and ornithinedecarboxylases were usually present, but one strain was lysinedecarboxylase negative and 6 strains were ornithinedecarboxylase negative

Acid production from the sugars in which different strains gave different results is shown in Table 2 The differences appear stable according to the repeated tests and have been used as a basis for a subdivision into 11 fermentation types arbitrarily designated "a' through 'k

The sensitivity tests mainly served to exclude multiresistance By the method used all strains were resistant to penicillin There were 5 strains totally resistant to sulphonamides, two totally resistant to streptomycin and 3 totally resistant to tetracycline Against streptomycin chloramphenicol, polymyxin B and ampicillin part of the strains showed intermediate degrees of sensitivity All strains were fully sensitive to gentamycin and kanamycin

It is thus apparent that the antibiotic sensitivity of these variants is not unusual in any way and they are not in general multiresistant Only one, strain, No 18, showed complete resistance against three of the antibiotics (sulphonamide, penicillin and ampicillin)

H_2S Production

The 26 isolates described in the present paper were at first established as hydrogensulphide positive variants by stab inoculation into tubed ferrous chloride gelatine medium

Formation of hydrogensulphide from sodium thiosulphate could regularly be demonstrated in individual colonies on the placenta thiosulphate ferric citrate medium by a central black sulphide precipitate provided there was sufficient spacing between the colonies The precipitate is visible in 24 hours even under aerobic conditions of incubation By transferring the plates to an anaerobic jar after 24 hours of aerobic incubation the anaerobic nature of the H S production was clearly manifested by a substantial enhancement of the black precipitate within a period of a few hours or less A similar medium made with meat infusion was unable regularly to give sufficient blackening under aerobic conditions whereas under anaerobic conditions H S production could be regularly demonstrated Probably the difference in H_2S production between meat infusion and placenta infusion agar under aerobic conditions can be explained by the more voluminous colonies on the latter medium giving more anaerobic conditions in the centres of the colonies and therefore more favourable conditions for the reduction

During subculture of the 26 H S positive *E coli* strains on the surface of the placenta infusion medium H S negative colonies occasionally appeared when the seeding was made from stock cultures A systematic quantitative study was not made but 5 H_2S negative cultures were selected from 5 originally positive ones

In order to characterize further the nature of the H S production a series of reduction tests was made in tubes containing the solid defined H S indicating growth medium with different sulphur compounds

The behaviour of the H_2S positive strains and their H S negative offspring and that of typical H_2S negative *E coli* when tested in the different media is shown in Table 3

The *E coli* strains recorded as H S positive in the ferrous chloride gelatine test are all able to form hydrogensulphide from thiosulphate and tetrathionate in contrast to typical *E coli* and the derived H S negative cultures The ability to form H S from cysteine, as well as the inability under the conditions used to reduce sulphate and sulphite to H S, are shared by all three categories of strains The results suggest that the H S positive *E coli* might possess at least two additional functional enzymes i e a tetrathionate reductase and a thiosulphate reductase

Serological Characteristics

Thirteen different O H serotypes were found among the 26 strains after determination of the O and H antigens (Table 2) Ten strains belonged to the same type, *i e* O8 O60 H51, three to O11 H52, two to O76 H34 and two to 'O90 H Two non motile strains Nos 19 and 21, of different fermentation types were both assigned to O group 8 One of them had to be autoclaved to react in the O antiserum, which means that a K antigen of the A type was present, this was not the case with the other strain so they are considered to be different One strain No 7 was auto agglutinable and is therefore denoted as rough, whereas another strain No 22 did not react in any O antiserum Since in both these cases the H antigens found were not represented among the other serotypes present, they are considered as separate serotypes

Since the two most frequent O serotypes O8 O60 and O11, with one exception possessed H antigens which did not react in *E coli* H antiserum new H antisera were prepared with one of the O8 O60 and one of the O11 strains No significant cross reactions between these new H antigens and the already established ones were found therefore they were given new numbers and H52

From Table 2 it is seen O8/ H51 was found in five out

TABLE 2 *Each H_2S Producing* E coli, *with Added Serol*

Number of strains	Acid formation from salicin	sucrose	dulcitol	raffinose	mannitol	sorbitol	sorbose	adonitol	Fermentation type	Geograph Roskilde	Gamles By
12	+	+	+	+	+	+	—	—	a	1)8 60 51 2)8,60 51	
2	+	+	+	+	+	+	+	—	b		
2	+	+	+	+	—	+	—	—	c		
1	+	+	—	+	+	+	—	—	d		
1	+	+	—	+	+	+	—	+	e		
3	+	—	—	—	+	+	—	—	f	3)11 52	5)11 52
1	+	—	—	—	+	—	—	—	g		
1	+	—	—	+	+	+	—	+	h		
1	+	—	+	—	+	+	—	—	i		
1	—	—	+	+	+	+	—	—	j		
1	—	+	+	+	+	+	—	—	k		

Figures in front of the serotype indicate strain number With the exception of strain 19 and 21 (see text), the strains were not examined for the presence of K antigens and are therefore listed as O H sero types, *e g* 11 52 means that the strain belongs to O group 11 and has H antigen 52 In some cases

two geographical areas The three O11 H52 strains originated from three different hospi tals in two geographical areas *E coli* strains belonging to O group 11 are rarely isolated, while O8,O60 strains are not uncommon and if the latter strains are motile they are almost always equipped with a known H antigen These facts seem to point to the likelihood of some epidemiological connection between the H S positive strains of type *O11 H52* and also between the O8,O60 H51 *strains*

The five isolates which spontaneously had lost the H_2S producing ability were examined for a change of O and H antigens Three of the strains were of the O8,O60 H51 type In no case had an antigen change taken place

Transfer of the H_2S Producing Character

The *E coli test strain of O antigen 100* and derivatives of this were known from *previous* experiments to be capable of receiv ing extrachromosomal elements (14) A streptomycin (W3479) or a streptomycin and *nalidixic acid resistant (D726)* mutant of this strain (O100) was therefore used as recipient in most experiments In five cases a streptomycin resistant K12 mutant, W1607, was used as recipient and in six cases transfer experiments were carried out with both the O100 and the K12 strain

Transmission of the H_2S producing capa city took place only with strain *9 (O69,O149 H–) as donor, in which case about* 90 per cent of W1607 and about 50 per cent of W3479 colonies had acquired this ability after 24 hours incubation W3479 recipients were examined for transfer of other char acters from the donor known to differ from those of the recipient (O antigen, non motil ity, ability to grow without *addition of histi* dine and isoleucine failure to ferment dulcitol *and sorbitol and resistance* to tetracycline) None of these characters were found *to be* transferred In particular it should be stressed that resistance to tetracycline was not trans

...a (A B & C) and hospitals						
A		B			C	
Rigshosp	Frederiksberg	Ålborg Nord	Ålborg Syd	Hjørring	Århus	Grenå
8 8 60 51	11)8 60 48	19)8 A –				
2)8 60 51	14)8,60 51				23)8 60 51 24)8 60 51 25)8 60 51 26)8 60 51	16)8 60 51
	17)76 34 18)76 34					
					13) 90 – 15) 90 –	
	22)? 21					
		10) 117				
				4 11 52		
			9 69 149 6 69			
		7)rough 9 20)53 16				
	21)8 A+					

...t was necessary to assign the strain to two O grou... ...rain I When the titre in a reacting O antiserum was too low for a definite assignment to the O ... quest on the number of the O group was inserted in quotation marks

...mitted as one might have expected if the transfer of the H S character was directed by a resistance transfer factor The efficienc... of transfer to W 3479 after 30 minutes mixed incubation was 0 3 × 10 ² The H-S character was further transferable from W 3479 to W1607 No transfer took place during a period of 30 minutes if the donor culture was filtered through a Millipore filter HA suggesting that phage involvement is rather unlikely in the transfer of the H_2S character from strain 9

The H S positive strains unable to tran... the H S character to W 3479 were retested ... W1607 as recipient but without suc...cess.

In order to determine whether the failure of transfer of the H-S character from the remaining 25 strains was due to loss of the transfer factor experiments were carried out to ascertain whether the R factor R ... carry... ing resistance to sulphonamide streptomycin chloramphenicol and tetracycline ... could

as carrier for the H_2S character ...fer of this R factor to strain 12 ... 0 H51) was successful and so was ...her transfer of the resistance deter... from strain 12 to the O100 recipient ...sistant to nalidixic acid However, ...000 recipient colonies which had ... resistance determinants did not ...he capacity to produce H S ...inary investigations into the question ...r the H S transfer factor demonstrated ...9 is of the F or the I type (11) ...hat it most probably is of the F type ... experiments are in progress and ...will be published later

DISCUSSION

I... ...ants must undoubtedly be identified ... E... ... Apart from the ability to produce H S they are in complete agree... ...ment with the pattern typical for *E coli* ... in addition many of the strains possess ...

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Acta path microbiol scand Section B 79, 651–664 1971

CELL DIVISION IN A CHAIN-FORMING *envA* MUTANT OF *ESCHERICHIA COLI* K12

Fine Structure of Division Sites and Effects of EDTA, Lysozyme and Ampicillin

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A chain forming mutant of *Escherichia coli* K12 has recently been described which exhibits a decreased tolerance to ampicillin, rifampicin actinomycin D, and several other anti bacterial agents (*Normark et al* 1969, *Normark* 1970) The mutated gene responsible for chain formation and drug sensitivity, denoted *envA*, was mapped at 1 5 min (*Normark* 1970) In this paper the cell division process in the *envA* mutant and its wild type parental strain is investigated Electron microscopy revealed that all layers of the cell envelope partici pate in the invagination process during cell division in the wild type strain In contrast in the chain forming *envA* mutant a septum was constantly formed at the site of division which sepa rated individual cell units The septum was delimited by the plasma membrane and was com posed of periplasm and a central septal structure which by its sensitivity to lysozyme was identi fied as the murein skeleton Plasmolysis of chains by 30 per cent sucrose caused a significant broadening of the septal regions but left the murein layer intact Ampicillin was without effect on the septal murein component Sites at which the cytoplasmic membrane was attached to the cell wall were frequently observed in close relation to the invagination areas, even after removal of the murein layer by lysozyme Inhibition of DNA and murein synthesis did not affect the ability of chains to be transformed into separate cells Protein synthesis however, was a prerequisite for cell separations It is suggested that the *envA* gene mediates a defect in the association of the murein skeleton with the outer layers of the envelope Models for septum formation are presented

The growth and division cycle of a rod shaped Gram negative bacterium involves a complex series of events A newly divided cell of *Escherichia coli* grows by elongation at specific growth sites (*Schwarz et al* 1969, *Donacchie* & *Begg*, 1970) According to these authors the active growth is limited to certain areas of the bacterium which can be identi fied by their special susceptibility to penicil lins The number of growth sites per cell may vary from one to two depending on the generation time of the organism At low growth rates the sites for growth and cell division coincide Furthermore, during a divi sion under these conditions a duplication of the growth sites occurs At high growth rates the zones for cell elongation and cell division are not necessarily identical

In the study of cellular functions the isola- tion of different mutants has turned out to be a very useful tool In most mutants with a defective cell division the invagination pro cess of the cell envelope is totally inhibited Such filament forming mutants often have a conditional blocking of the invagination pro cess which is correlated to an impaired DNA

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synthesis (*Howard-Flanders et al* 1964, *Hirota et al* 1968) However, *Normark et al* (1969) have described a mutant of *E coli* K12 which formed chains of cells at high growth rates Examination of this mutant in the light microscope revealed that it exhibited distinct invaginations and was therefore morphologically different from filament-forming strains Conjugation showed that the mutated gene, designated *envA*, was located at 2 4 minutes (*Normark et al* 1969) A further transduction analysis has shown the *envA* gene to be cotransducible with *leu* and *azi* and thus located at 1 5 min (*Normark* 1970)

Recent electron microscopic investigations of the *E coli* cell envelope have clearly demonstrated that this structure consists of an inner and an outer triple layered membrane and an intermediary structure, identified as the murein skeleton (*Murray et al* 1965, *de Petris* 1965, 1967, *Naninga* 1970) During a normal cell division all layers participate in the invagination process Light miscroscopic observations on our *envA* strain had occasionally shown that chains could contain units which had been transformed to ghosts This suggested the existence of a barrier between different cell units Our present work has shown that each cell unit of a chain is actually separated by the plasma membrane as well as by a structure which includes what we consider to be the murein skeleton

MATERIAL AND METHODS

Bacterial strains Both the *envA* mutant, D22, and its parental strain, D21, have been described previously (*Normark et al* 1969) Both strains contain the *ampA* gene at *82* min which mediates a 10 fold increase in the amount of penicillinase produced by each cell This gene was replaced by its wild type allele in a recombinant, strain D220 as described by *Normark* (1970) Strain D22a12 was a recombinant obtained from strain D22 The genotype *is believed to be ampA plus a* defect in the structural gene for the chromosomal penicillinase (*pblA*) The donor strain with the *pblA8* allele was isolated by Burman (unpublished)

Medium and growth conditions Bacteria were grown in the LB medium of *Bertani* (1951) supplemented with 0 2 per cent glucose and the basal medium E of *Vogel and Bonner* (1956) Cells were grown for several generations at a maximum growth rate and were harvested at densities of 2 4×10⁸ cells/ml

Materials D ampicillin was kindly provided by AB Astra, Sodertalje, Sweden, chloramphenicol was obtained from Erco, Stockholm, Sweden, nalidixic acid was purchased from Winthrop Ltd Surbiton on Thames, England and lysozyme, 3 × crystallized, was obtained from Sigma Chemical Co St Louis Missouri USA

Determination of cell size Cell size distribution was determined in a Coulter counter model B equipped with a 70 μm diameter aperture Calibration material with known particle diameter was obtained from Coulter Electronics Ltd, Dunstable England A lower threshold setting of 5 an aperture current setting of 1/374, and an amplification setting of 1/4 corresponded to a particle volume of 2 3 μm^3 This size was found to be slightly larger than the average volume of rapidly growing cells of normal *E coli* strains Total cell count was obtained with a lower threshold setting of 1

Electron microscopy All growing bacteria were immediately chilled in ice water, centrifuged and the pellets were fixed in ice cold 4 per cent glutaraldehyde in 0 1 M phosphate buffer (pH 7 3) for 2 hours (*Sabatini et al* 1963) The specimens were thereafter rinsed for one hour at 4°C in 0 1 M phosphate buffer containing 0 2 M sucrose They were then post fixed for an additional hour at 4°C in 1 per cent osmium tetroxide dissolved in the sucrose containing buffer (pH 7 3) and thereafter rinsed again, this time in distilled water for one hour Dehydration of all specimens was carried out stepwise in ethanol at room temperature and embedding was performed in Epon 812 according the the method of *Luft* (1961) The blocks were cured at 37°C overnight and for an additional period of 24 hours at 60°C Sections were cut on an LKB Ultrotome using glass knives Thin sections for electron microscopy were post stained with uranyl acetate and lead citrate and were examined in a Philips EM 300 electron miscroscope

When cells were plasmolyzed the chilled culture was divided into aliquots which were centrifuged and resuspended in ice cold 30 per cent sucrose in 0 05 M Tris HCl (pH 7 4 or 8 0) After the various treatments described in *Results* the cells were centrifuged and fixed for two hours in ice cold 4 per cent glutaraldehyde in 0 1 M phosphate buffer (pH 7 3) containing 30 per cent sucrose All further processing was carried out as described above

RESULTS

Comparative morphology of strains D21 and D22 Size distribution was separately

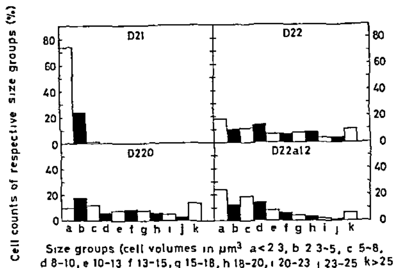


Fig 1 Cell size distribution of strains D21, D22, the *ampA*$^{+}$, *envA* strain D220 and the *pblA, ampA, envA* containing strain D22a12 The strains were grown overnight in LB medium diluted 10^{-2} with pre warmed LB and incubated at 37°C on a rotary shaker to a Klett value of 100 corresponding to about 4×10^{8} cells/ml The cultures were immediately chilled, and diluted in 10 ml 0 15 M NaCl Counts were read at different threshold values in a Coulter counter, model B

Fig 2 Survey electron micrograph of the parental strain D21 The cells show a normal binary division × 7 800



Fig 3 Survey electron micrograph of the *envA* mutant D22 Four chains containing several cell units are seen $\times$ 7,800

investigated for each of our strains The results in Fig 1 show that in strain D21 75 per cent of the cells were smaller than 2 3 μm^3, whereas 25 per cent were in the size group 2 3 5 μm^3 In the *envA* mutant D22, size distribution was markedly different About 15 per cent of the cells were smaller than 2 3 μm^3 while a variety of larger units were present The average length corresponded to about four to five cell units per chain, assuming that the diameters of the two strains are approximately the same As both strain D21 and D22 contain the regulatory and the structural genes for chromosomal penicillinase, we also compared the size distribution of strains D220 and D22a12 These strains are believed to be wild type in the regulatory gene and deficient in the structural gene for penicillinase, respectively The results in Fig 1 show that neither of these genes had any significant influence on size distribution

Figs 2 and 3 show survey electron micrographs of strains D21 and D22, respectively The parent strain D21 was characterized by a normal binary cell division and no bacterium was observed producing more than two daughter cells This is in contrast to the *envA* mutant, D22, which regularly formed chains with varying numbers of cell units which were incompletely separated from each other The length and diameter of individual cell units in both strains was found to be about 2 5 μm and 1 1 μm respectively This gives a cell volume of about 2 μm^3

Examination of the cell division sites in the two strains at higher magnification revealed the following characteristic details In the parental strain, D21, the cell division proceeded as a concentric inward growth of all layers of the cell envelope (Fig 4) In this strain no structures were ever observed between the opposed invaginations In contrast, a septum was constantly seen connecting the two invaginations in the *envA* containing strain D22 (Fig 5) Each cell unit is delimited from the septum by the *plasma*



ıg 4 Cell division site in the parental strain D21 All layers of the envelope participate in the concentric invagination process ×62,400

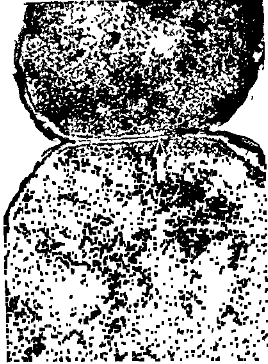


Fig 5 Cell division site in the *envA* mutant D22 A septum separates individual cell units Centrally located in the septum is a thin structure of moderately electron dense material Close to one invagination point this structure is split into two components (arrow) ×60,200

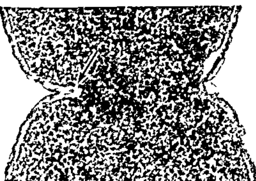


Fig 6 Early stage in formation of a septum in strain D22 Arrow indicates a fold of the intermediate layer of the cell envelope, invaginating into the forming septum × 61,700



Fig 7 Somewhat later stage in septum formation in strain D22 The electron lucid septal ingrowth originates at opposing invagination points In the center of each ingrowth a delicate strand of material is observed × 61,600

membrane In the middle of the septum it is possible to discern a thin structure which near each invagination site separates into two components In numerous micrographs is has been possible to follow the latter structures and establish that they constitute a part of the normal cell envelope (see Fig 8) A comparison of micrographs of our two strains D21 and D22 indicates that the latter mutant carries some defect in the outer layers of the

for 90 min and then added to 500 μl human serum The volume was adjusted to 5 0 ml with VBS and the sample was incubated at 4°C for 19 hr In this experiment 14 of 37 CH_{50} units were inactivated If guinea pig IgG was substituted for human IgG in an identical experiment 17 CH_{50} were inactivated

DISCUSSION

The addition of protein A to human serum decreases the complement titre as it does the complement titre of guinea pig serum (16) The consumption of complement increases as increasing amounts of protein A are added Maximal C fixation is obtained by addition of 125–250 μg protein A to 500 μl fresh human serum Approximately 70 per cent of the complement available is then inactivated

With human complement as with guinea pig complement (16), maximal fixation occurs in the region of 'antibody excess as judged by the precipitation curve This was also recently noted by *Kronvall* & *Gewur* (10) However, these authors did not observe any complement fixation when human serum was incubated at 37°C for 60 min with amounts of protein A giving maximal precipitation with serum after 1 week at 4°C The differences in procedure between their work and ours can account for the discrepancies in the results

There is considerable variation in the amounts of human complement fixed at different temperatures Maximal C fixation at 4°C for 19 hr is only 50 per cent of that at 37°C for 90 min At 0°C no C fixation is found (10) The fixation of complement to antigen antibody complexes is known to be temperature dependent (14) The amounts of IgG precipitated from human serum by protein A at 4°C and at 37°C are similar

Thus the differences in complement fixation can not be explained by different yields of protein A IgG aggregates at the two temperatures Since the complement titre of the serum is not influenced by the incubation temperatures used the phenomenon must depend on differences either in the structures of the protein A IgG complexes or in the kinetics of the complement inactivation steps involved Experiments with aggregates formed at 37°C and incubated with human serum at 4°C support the possibility that the conformation of the protein A IgG aggregates is the important factor in complement fixation Thus, aggregates of protein A IgG preformed at 37°C inactivated 65 per cent of the available human complement at 4°C compared with 38 per cent and 35 per cent inactivation which occured when equal amounts of aggregates were formed at 4°C Preformed aggregates of protein A and human IgG fix human complement at 4°C as well as does a mixture of the same amount of protein A and human serum incubated at 37°C for 90 min (Fig 1)

These results support the hypothesis that the complexes formed between protein A and IgG at different temperatures are qualitatively different and that variations in complement fixation are due to these differences

Guinea pig serum differs from human serum with respect to temperature dependence Guinea pig complement is readily inactivated by protein A at 4°C

This difference may depend on differences between the complement systems of the two types of sera or the immunoglobulins of the two species may differ with respect to their aggregability by protein A The latter possibility is supported by the fact that guinea pig IgG added to human serum is able to augment complement fixation by protein A at 4°C Human IgG does not have this effect The problem may be solved using purified complement components

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TABLE 3 *Fixation of Rabbit Complement by Protein A or by Protein A and Human IgG**

	RC	RC + 1 25 mg protein A	RC + 6 75 mg HIgG + 1 25 mg protein A	RC + 6 75 mg HIgG
CH_{50}	29	22	22	28

* For experimental conditions see text

mg protein A to 150 μl serum, no definite *maximum point* is obtained The maximum may rather be a plateau This differs from the results obtained with serum of other species Rabbit serum differs from the other sera used in other respects as well For example, it does not form a precipitate with protein A The addition of 6 75 mg human IgG to 1 5 ml serum, followed by addition of 1 25 mg protein A, results in a precipitate, but C fixation is not increased (Table 3) This indicates that the comparatively small fixation of rabbit complement depends on differences between the complement systems in rabbit and man The failure of protein A to produce the Arthus reaction in untreated rabbits should then depend on other factors For example the formation of soluble complexes of protein A and rabbit IgG *in vivo* which are promptly removed from the site of injection of protein A may be the explanation

It is possible to inhibit complement fixation by IgG protein A complexes by the addition of EDTA to the system This is what would be expected if the complement inactivation occurs via C1, as in an ordinary antigen antibody system However, *Sandberg et al* (15) found that inactivation of complement by another pathway can be inhibited by EDTA Preformed aggregates of antigen and guinea pig IgG1 antibody inactivate the complement chain from C3 and this is prevented by EDTA According to *Kronvall* & *Gewurz* (10) protein A inactivates even the early acting components of complement This will be examined by the use of purified complement components

The author is indebted to Professor *J Sjoquist* for helpful criticism and discussion The skilful technical assistance by Mrs *L Moller* and the secretarial work done by Mrs *M Gustafson* is also gratefully acknowledged

This work was supported by grants from the *Swedish Medical Research Council* and the *Swedish Cancer Society*

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for 90 min and then added to 500 μl human serum The volume was adjusted to 5 0 ml with VBS and the sample was incubated at 4°C for 19 hr In this experiment 14 of 37 CH_{50} units were inactivated If guinea pig IgG was substituted for human IgG in an identical experiment, 17 CH_{50} were inactivated

DISCUSSION

The addition of protein A to human serum decreases the complement titre as it does the complement titre of guinea pig serum (16) The consumption of complement increases as increasing amounts of protein A are added Maximal C fixation is obtained by addition of 125 250 μg protein A to 500 μl fresh human serum Approximately 70 per cent of the complement available is then inactivated

With human complement, as with guinea pig complement (16), maximal fixation occurs in the region of 'antibody excess' as judged by the precipitation curve This was also recently noted by *Kronvall & Gewurz* (10) However, these authors did not observe any complement fixation when human serum was incubated at 37°C for 60 min with amounts of protein A giving maximal precipitation with serum after 1 week at 4°C The differences in procedure between their work and ours can account for the discrepancies in the results

There is considerable variation in the amounts of human complement fixed at different temperatures Maximal C fixation at 4°C for 19 hr is only 30 per cent of that at 37°C for 90 min At 0°C no C fixation is found (10) The fixation of complement to antigen antibody complexes is known to be temperature dependent (14) The amounts of IgG precipitated from human serum by protein A at 4°C and at 37°C are similar

Thus the differences in complement fixation can not be explained by different yields of protein A–IgG aggregates at the two temperatures Since the complement titre of the serum is not influenced by the incubation temperatures used, the phenomenon must depend on differences either in the structures of the protein A–IgG complexes or in the kinetics of the complement inactivation steps involved Experiments with aggregates formed at 37°C and incubated with human serum at 4°C support the possibility that the conformation of the protein A–IgG aggregates is the important factor in complement fixation Thus, aggregates of protein A–IgG preformed at 37°C inactivated 65 per cent of the available human complement at 4°C compared with 38 per cent and 35 per cent inactivation which occured when equal amounts of aggregates were formed at 4°C Preformed aggregates of protein A and human IgG fix human complement at 4°C as well as does a mixture of the same amount of protein A and human serum incubated at 37°C for 90 min (Fig 1)

These results support the hypothesis that the complexes formed between protein A and IgG at different temperatures are qualitatively different and that variations in complement fixation are due to these differences

Guinea pig serum differs from human serum with respect to temperature dependence Guinea pig complement is readily inactivated by protein A at 4°C

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The authors [illegible] for helpful comments [illegible] technical assistance [illegible] secretarial work done by [illegible] gratefully acknowledged

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Acta path microbiol scand Section B 79, 673–678, 1971

RAPID IDENTIFICATION OF PROMPT LACTOSE-FERMENTING GENERA WITHIN THE FAMILY *ENTEROBACTERIACEAE*

O Closs and A Digranes

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Department of Microbiology, Bergen Norway

Two hundred and two strains of prompt lactose fermenting enterobacteria were examined, using 12 biochemical tests In addition the susceptibility to several antibiotics was recorded Identification on the basis of H_2S production ornithine decarboxylase activity, motility and citrate utilization (HOMoC) was compared with the results obtained using the classical IMViC tests indol, methyl red, Voges Proskauer and citrate Only the former set of reactions seemed suitable for clear cut identification of the 4 genera *Escherichia, Citrobacter, Enterobacter* and *Klebsiella* In showing low sensitivity to cephalotin *Citrobacter* and *Enterobacter* seemed to differ from the other 2 genera, and this could be used as an additional criterion for their identification

The high frequency of the prompt lactose-fermenting members of the family *Enterobacteriaceae* (LFE), especially as a cause of urinary tract infection, has made these bacteria appear so trivial to many laboratory workers that their proper identification has been neglected To a large extent the identification of LFE has been based on colony morphology and other subjective criteria In the opinion of *Steel* (15) this is unsatisfactory The characters used should be independent of subjective criteria such as rate of growth, odour and the finer shades of pigment, " In other instances certain biochemical tests are carried out the indol, methyl red, Voges-Proskauer and citrate reactions (IMViC) constituting the standard scheme The importance of a more precise classification of the members of this group of bacteria has recently been stressed by several authors (1, 13, 17, 18)

According to recent nomenclature (9, 14) LFE consists of the four genera *Escherichia, Enterobacter, Klebsiella* and *Citrobacter* It seems difficult to identify these genera on the basis of the reactions in the IMViC scheme We have therefore been in search of [illegible]

tification on the basis of H_2S production, ornithine decarboxylase activity and citrate utilization (HOC) has previously been proposed (17)

The present investigation was carried out to select the set of reactions best suited for routine work

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According to recent nomenclature (9 14) LFE consists of the four genera *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter* It seems difficult to identify these genera on the basis of the reactions in the IMViC scheme We have therefore been in search of another set of reactions equally limited in number but permitting a more clear-cut distinction Identification on the basis of H S production ornithine decarboxylase activity and utilization (HOC) has previously ... posed (17)

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TABLE

	No of Strains	Ind +	Ind —	M–R +	M–R —	V–P +	V–P —	Cit +	Cit —	ODA +	ODA —
Escherichia	115	115	0	115	0	0	115	0	115	83	32
Citrobacter	26	5	21	25	1	1	25	21	5	3	23
Klebsiella	35	8	27	9	26	26	9	35	0	0	35
Enterobacter	26	1	25	4	22	22	4	26	0	25	

Ind = indol
M R = methyl red
V–P = Voges Proskauer
Cit = citrate
ODA = ornithine decarboxylase

MATERIALS AND METHODS

Strains All strains included in this study were isolated from patients with urinary tract infections The material consisted of 202 strains isolated from August 1969 to July 1970 Primary isolation was made on nutrient agar plates containing 1 per cent lactose, 0 1 per cent sodiumthiosulfate, 0 005 per cent crystal violet, 0 008 per cent bromthymol blue Colonies of prompt lactose fermenting strains were picked from these plates to obtain pure cultures

Identification Criteria for identification of strains were taken from *Cowan & Steel* (3) and *Edwards & Ewing* (6)

Media and reagents Indol production (1 per cent Bacto tryptone, Kovacs' reagent), methyl red and Voges Proskauer reactions (0 5 per cent Bacto-peptone, 0 5 per cent K_2HPO_4, 0 5 per cent glucose in aq dest using α naphtol and KOH for the detection of acetoin (3, p 160), citrate utilization (Koser's medium with 0 5 per cent sodium citrate), ornithine decarboxylase activity (ODA) (Decarboxylase Medium (Difco) with 1 per cent ornithine and 0 3 per cent agar (10)), H_2S production (nutrient agar containing 0 2 per cent lead acetate), motility test (motility medium of *Edwards & Bruner* (5) with 0 2 per cent sodium citrate), urease activity (2), KCN test (12), fermentation of glucose, inositol and arabinose (meat extract broth containing 1 per cent carbohydrate and bromthymol blue indicator) All tests, with the exception of the methyl red and Voges Proskauer reactions, were read after overnight incubation at 37°C These 2 reactions were read after 48 hours (14) If a test was difficult to read it was repeated or the incubation was continued for another 24 hours The sensitivity of the strains to ampicillin, sulfaisodimidine, cephalotin, nitrofurantoin, tetracycline, nalidixic acid, kanamycin and colistin was tested, using the disc diffusion method (7)

RESULTS

Biochemical reactions The biochemical reactions of all the strains are given in Table 1 Each strain was identified on the basis of all reactions The strains showing an atypical reaction pattern are discussed below

Escherichia Two strains (1 7 per cent) did not produce gas from glucose, and 1 strain (0 9 per cent) showed growth in KCN This is in accordance with data given by *Edwards & Ewing* (6)

Citrobacter Five strains (19 2 per cent) produced indol One of these did not utilize citrate, 1 was ODA positive and did not produce gas from glucose, 3 did not grow in KCN Of the other strains identified as *Citrobacter* 4 did not utilize citrate, 2 were ODA positive and 1 strain was KCN negative Eighteen of our strains (69 3 per cent) produced urease which accords well with previous findings (3, 4)

Klebsiella Eight strains (22 8 per cent) were indol positive, none of them showing other aberrations in the reactions pattern One strain (2 9 per cent) did not produce urease, and another strain was KCN negative

Enterobacter One strain (3 9 per cent) produced indol and was also methyl red positive and Voges-Proskauer negative Three strains were KCN negative but showed no other atypical reactions Three strains (11 8 per cent) were non motile, this incidence being similar to that reported by others (11 16, 18) Only 1 strain (3 9 per cent) was ODA negative

After having identified the 202 strains ac

H₂S +	H₂S —	Mot +	Mot —	Ur +	Ur —	KCN +	KCN —	Arab +	Arab —	Ino +	Ino —	Gluc +	Gluc —
0	115	74	41	0	115	1	114	115	0	0	115	113	2
26	0	25	1	19	7	23	3	26	0	0	26	25	1
0	35	0	35	34	1	34	1	35	0	28	7	33	2
0	26	23	3	14	12	22	4	26	0	7	19	26	0

Mot = motility
Ur – urease
Arab – arabinose
Ino = inositol
Gluc = gas from glucose

TABLE 2 *Reaction Pattern of 202 Strains of LFE in the HOMoC Scheme*

H	O	Mo	C	Number of Strains within Each Genus
—	+	+	—	62 *Escherichia*
—	+	—	—	21 *Escherichia*
—	—	—	—	20 *Escherichia*
—	—	+	—	12 *Escherichia*
—	—	—	+	35 *Klebsiella*
+	—	+	+	17 *Citrobacter*
+	—	+	—	5 *Citrobacter*
+	+	+	+	3 *Citrobacter*
+	—	—	+	1 *Citrobacter*
—	+	+	+	22 *Enterobacter*
—	+	—	+	3 *Enterobacter*
—	—	+	+	1 *Enterobacter*

H – hydrogen sulfide
O – ornithine decarboxylase
Mo motility
C – citrate

cording to their pattern in the above 12 reactions we tried to find the minimum number of reactions giving an equally clear distinction Similarly to *Wolfe & Amsterdam* (17) we found that H_2S production, ornithine decarboxylase activity and citrate utilization constitute a good basis for differentiation between the 4 genera of LFE However, it has recently been reported that as many as 6–8 per cent of *Enterobacter* strains may be ODA negative after overnight incubation (16, 18) Although non motile strains of *Enterobacter* have been found with a frequency of 6–12 per cent (11, 16, 18) there seems to be no increased tendency for the non motile strains to be ODA negative or *vice versa* We therefore included a test for motility in the scheme The distribution of our 202 strains according to this identification scheme is given in Table 2 Of the 12 characters included in this study, no other com-

TABLE 3 *Reaction Pattern of 202 Strains of LFE in the IMViC Scheme*

I	M	V	C	Number of Strains within Each Genus
+	+	—	—	115 *Escherichia* 1 *Citrobacter*
—	—	+	+	18 *Klebsiella*, 22 *Enterobacter*
+	—	+	+	8 *Klebsiella*
—	+	—	+	9 *Klebsiella*, 17 *Citrobacter*, 3 *Enterobacter*
+	+	—	+	4 *Citrobacter*, 1 *Enterobacter*
—	+	—	—	3 *Citrobacter*
—	—	+	—	1 *Citrobacter*

I = indol
M – methyl red
V Voges Proskauer
C = citrate

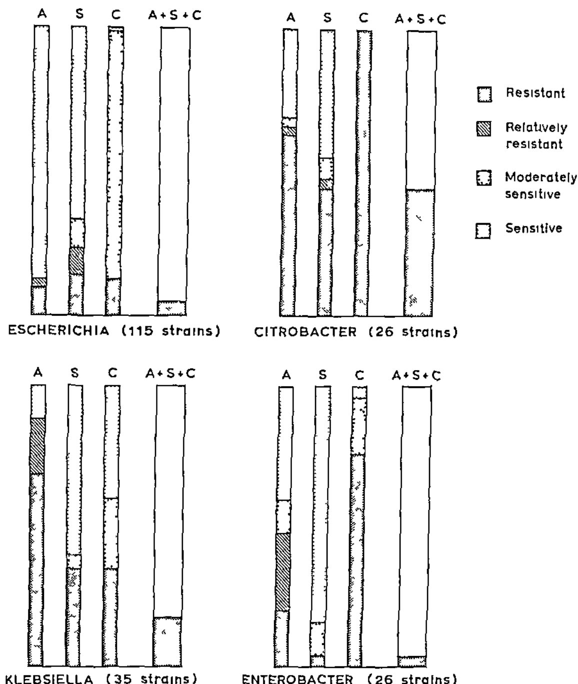


Fig 1 Sensitivity of *Escherichia Klebsiella Citrobacter* and *Enterobacter* to ampicillin (A) sulfasod midin (S) and cephalotin (C)

Sensitive means minimal inhibitory concentration <0.5 µg/ml (A) <2.5 mg/ml (S) and <2 µg/ml (C)
Resistant means minimal inhibitory concentration >150 µg/ml (A) >25 mg/ml (S) and >100 µg/ml (C)

bination constituted a better basis for identification

To demonstrate the insufficiency of the IMViC reactions in discriminating between the 4 genera of LFE we have included Table 3

Antibiotic sensitivity testing Some authors have recommended the use of antibiograms as an adjuvant to biochemical characterization (13 18) Fig 1 shows the sensitivity of the 202 strains to sulfaisodimidine ampicillin and cephalotin The sensitivity to the other antibiotics and chemotherapeutics tested showed no definite pattern corresponding to the different genera and these results have therefore been omitted All 26 strains of *Citrobacter* and most of the *Enterobacter* strains (77 per cent) were cephalotin resistant whereas most of the *Klebsiella* strains (66 per cent) were sensitive To our surprise the highest frequency of multiresistant strains was found among the citrobacters (46 2 per cent) and not among the klebsiellae (17 per cent)

DISCUSSION

Identification of bacterial isolates is a fundamental task for any bacteriological laboratory It is apparent from the data presented here (Table 3) that the 4 genera *Escherichia Citrobacter Enterobacter* and *Klebsiella* can not be clearly identified on the basis of the tests included in the IMViC scheme This has also been pointed out by others (17)

On the other hand by using the HOC scheme proposed by *Wolfe & Amsterdam* (17) all except one of our isolates an ODA negative *Enterobacter* strain could be readily identified To minimize the source of error due to ODA negative *Enterobacter* strains we have included motility as a fourth character our scheme thus bearing the designation HOMoC This scheme permits a greater extent of differentiation within some genera e g the isolates belonging to the genus *Escherichia* have 4 different reaction patterns (Table 2)

It is important that the characters which are selected as the basis of routine diagnostic work can be established by tests which are reliable and convenient to read With the possible exception of Koser's citrate test which requires a certain amount of experience to inoculate and to read correctly, all the tests in the HOMoC scheme seem to be highly suitable in this respect.

The use of the antibiogram seems to be of limited value as an adjuvant to the identification of LFE However cephalotin sensitivity can help in differentiating between *Klebsiella* and *Enterobacter* With the exception of multiresistant strains, *Klebsiella* is often inhibited by low concentrations of cephalosporin antibiotics whereas *Enterobacter* seldom is (18) We would thus suspect any strain which is ODA positive, non motile and cephalosporin resistant of being an *Enterobacter*

In our material none of the *Citrobacter* strains was inhibited by 200 mcg/ml of cephalotin or less We would therefore not readily accept any strain as *Citrobacter* unless it is also cephalotin resistant Furthermore citrate negative late H_2S producing strains of *Citrobacter* may be wrongly identified as *Escherichia* when using the HOMoC scheme Lack of sensitivity to cephalotin would indicate that the strain might belong to *Citrobacter*

We do not pretend to have all genera with possible lactose fermenting members equally well represented among our strains Members of some other genera might not as easily be correctly identified using the HOMoC scheme The Arizona group would according to our scheme erroneously be identified as *Citrobacter* but since no member of the Arizona group has ever been isolated in our laboratory we felt no need to includ[illegible] special tests for their identification Likew[illegible] following the HOMoC scheme, *Serr*[illegible] would be identified either as *Enterobact*[illegible] to *Citrobacter* According to *Edwards* [illegible] *Serratia* species ferment l[illegible] at all and we have so far [illegible] our prompt lactose fe[illegible]

As compared to mo[illegible] ods of identification

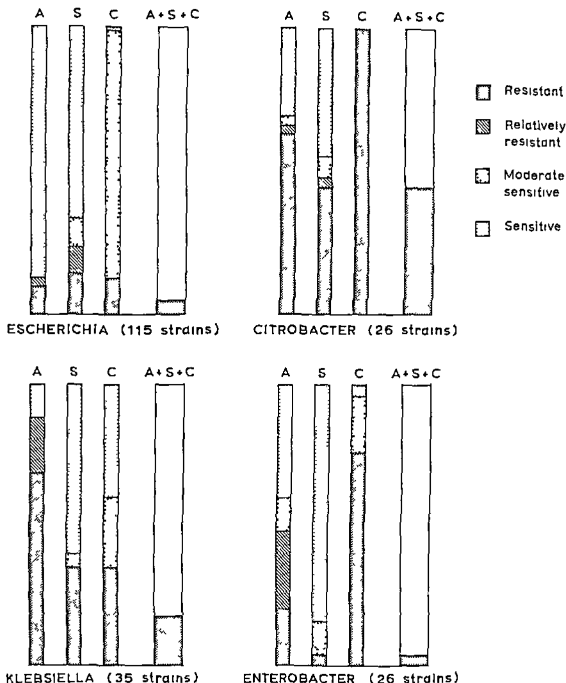


Fig 1 Sensit vity of *Escherichia Klebsiella Citrobacter* and *Enterobacter* to ampicillin (A) *sodimidin (S) and cephalotin (C)*
Sensitive means minimal inhib tory concentration <0 5 µg/ml (A) <2 5 mg/ml (S) and <2 µg/n
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Acta path microbiol scand Section B 79, 679–685, 1971

RUBELLA HAEMAGGLUTINATION INHIBITORS: THEIR SEPARATION FROM ANTIBODIES

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The separation of rubella haemagglutination inhibition (HI) antibodies and haemagglutination (HA) inhibitors by hydroxyl apatite chromatography and sucrose gradient centrifugation has been studied Both methods permitted inhibitors and antibodies to be demonstrated separately In hydroxyl apatite chromatography all inhibitors were found in the β lipoprotein fractions The usefulness of these methods in cases of suspected false positive and false negative reactions is discussed

The haemagglutination inhibition (HI) test for rubella antibody developed by *Stewart et al* in 1967 has proved to be an important tool in the serological diagnosis of rubella The HI test is more sensitive than the complement fixation fluorescent antibody and neutralization tests (8 10), and is highly specific (16) provided normal rubella haemagglutination (HA) inhibitors are removed from the serum The rubella HA inhibitors have been shown to belong to the β lipoproteins (6)

The treatment of serum with kaolin or with heparin $MnCl_2$ is widely used for the removal of nonspecific inhibitors of rubella HA. Recently another method employing dextran sulphate $CaCl_2$ has been described (11)

Considering the serious implications of the rubella diagnosis in early pregnancy, any method must ensure complete removal of inhibitors with minimal loss of antibody Contrasting views have been presented as to which method fulfills these requirements, and it has been claimed that false negative and false positive reactions do occur (3, 4, 5, 6, 11, 14, 15)

In an extensive study of the variables in the rubella HI test, *Schmidt & Lennette* (1970) present convincing evidence that the differing results obtained with kaolin treatment can be explained by the different pH values of the kaolin suspensions used When kaolin was suspended in a borate buffer at pH 9 0 absorption af antibody was minimized, and inhibitors were efficiently removed No distinction between IgG and IgM was made in this study, but from investigations made by others (6, 12) they assume that early appearing IgM may be absorbed by kaolin

The suspicion of false positive or negative reactions is based on comparison of HI titres obtained by different pretreatments of sera, from the results of other tests for rubella antibody, or from the patient's history In most cases a fin [illegible] to [illegible] que[illegible] will require the

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cribed here sacrifices a small degree of accuracy for savings in time and laboratory resources It nevertheless seems to us to be far better than any identification based on colony morphology and a certain number of biochemical tests more or less randomly chosen Correct identification of genera seems to us to be a modest aim when dealing with a group of bacteria which is the commonest cause of urinary tract infections The HOMoC scheme ought to make it an easy task for any bacteriologist to identify LFE genera according to the accepted nomenclature However, when *identification of species within the* genera is needed, further tests will of course have to be included

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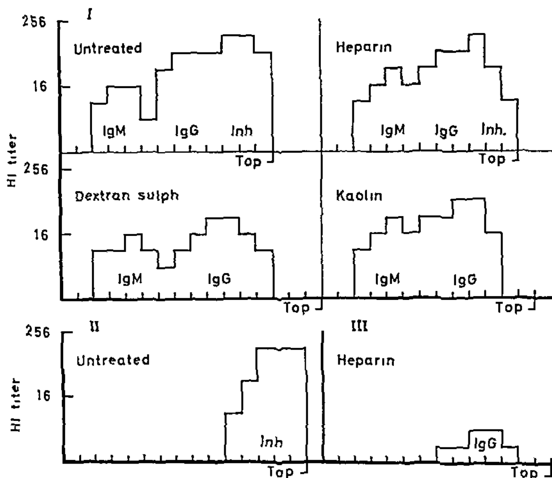


Fig 3 Sucrose gradient centrifugation I Serum 318/69 from a patient with clinical rubella treated as indicated *II Serum 898/70 from a 2 year old boy with a low HI titer III* Serum 896/70 from a sero-negative subject treated as indicated

float and can be recovered in the top fractions, *which should be free of antibody activity* Fig 3 shows that there was no indication of absorption of IgM or IgG rubella HI antibodies by any treatment It is seen that the inhibitors were removed by treatment with kaolin and with dextran sulphate $CaCl_2$, but not by the heparin $MnCl_2$ method of *Mann et al* (12) as mentioned above

Serum 898/70 illustrates the ultracentrifugation patterns of a serum with no rubella HI antibody Although the highest HI titers were found in the top fractions, there is some overlapping into the fractions which usually contain IgG antibodies

Serum 896/70 was obtained from a 2 year old boy with a low HI titer (20 after kaolin, and 40 after heparin (4) treatment) Usually the sero positives at this age show higher titers, and the serum was therefore checked for residual inhibitors It is seen that a typical IgG pattern was obtained The top fractions are free of HI activity, and residual non specific inhibitors can therefore be excluded

DISCUSSION

A recently published investigation of variables in the rubella HI test (14) has clearly shown the need for a strictly standardized technique It is of particular importance to avoid false positive reactions resulting from inadequate

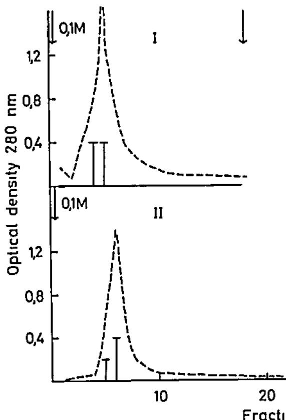


Fig 2 Hydroxyl apatite chromatography of a serum ι
Serum A D, diluted 1 10 before chromatography II Seru:
tion 1 10 Symbols --- Optical density at 280 nm The ν
titer

TABLE 1 *HI Titer of Fractions from Hydroxyl Apatite Chromatography (cf Fig 1) Before and After Treatment with Kaolin or Heparin Mn Cl_2*

Fraction	un treated	kaolin treated	heparin $MnCl_2$-treated
5	40	40	40
6	40	40	20
7	40	10	20
8	10	<10	<10
26 27*	40	<10	40
28	20	<10	20
35	2560	<10	<10
36	5120	<10	<10
37	40	<10	<10
38	10	<10	<10

* Fractions 26 and 27 were not separated during fraction collection

In the next
ing rubella anti
rum A D) was t
being applied to a h
A control column was r
as for kaolin treatment (
peaks were considerably low
with kaolin, but there was no
duction of the rubella HI titers
tions of the first two peaks As t
β-lipoproteins in the third peak we
tively removed by kaolin

Sucrose gradient centrifugation A se (318/69) obtained from a patient with cli ical rubella was centrifuged in a sucrose gradient to examine the effect of kaolin heparin $MnCl_2$ and dextran sulphate $CaCl_2$ treatments on IgM and IgG rubella HI antibodies and on the inhibitors present In the sucrose gradient the light β lipoproteins will

are not significantly reduced Investigations are in progress to further elucidate these observations

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removal of inhibitors because of the serious consequences for the pregnant woman when her immune status is determined

A weak point in the many reports of false positive reactions is that the alleged presence of residual inhibitors is not confirmed by separation of antibodies and inhibitors Several methods are available for separation of serum components such as sucrose gradient centrifugation hydroxyl apatite chromatography gel filtration DEAE cellulose chromatography and immunoelectrophoresis The latter method is not sensitive enough for the present purpose and gel filtration and DEAE cellulose will not separate macroglobulins from β lipoproteins We have therefore examined the usefulness of sucrose gradient centrifugation and hydroxyl apatite chromatography

On sucrose gradient centrifugation there is some overlapping between IgG and β lipoproteins (*Fig 3*) but their peaks are well separated When rubella IgG HI antibodies are not present residual inhibitors in pretreated sera can easily be demonstrated Likewise low titered rubella HI antibodies are readily distinguished from possible residual inhibitors

The best method for separation of the inhibitors is beyond doubt chromatography on hydroxyl apatite In our experiments the β lipoprotein inhibitors were completely separated from HI antibodies and no inhibitors were found outside the β lipoprotein fractions Recovery of proteins from hydroxyl *apatite columns is very high (9) and the* inhibitors are eluted as a sharp peak which will permit the detection of a possible small residual quantity (Figs 1 and 2) When stepwise elution is employed a column can be run in the course of 4 to 8 hours and will provide a definite answer in urgent cases When small amounts of rubella HI antibodies are suspected untreated and undiluted serum can be applied to the column and the 0 1 M elution can be omitted

No false positive reactions were found in our limited selected material or in our routine testing of clinical specimens However a false positive reaction may of course have occurred in our routine material and would only have been suspected if a positive HI test had been obtained where an earlier exposure to rubella was unlikely This situation may occur in children as illustrated in Fig 3 since a recent rubella infection usually leaves a high antibody titer In adults a low HI titer prior to a rubella infection does not necessarily rule out antibodies as re infections have been shown to occur

Another question of some controversy is whether rubella HI antibodies are removed together with inhibitors Several investigators have claimed that this occurs with kaolin treatment (3 5 6 11 12) In recent studies however *Herrmann et al* (8) examined sera from children with inhibitor titers lower than antibody titers They showed that rubella HI antibody titers were not significantly reduced by kaolin treatment Their results are in agreement with those of other investigators (2 8 10 14) and the present findings

Cabasso et al (2) presented evidence that rubella HI antibodies in serum are protected by albumin against absorption by kaolin In our experiments this may explain why some rubella HI antibodies eluted from hydroxyl apatite after the albumin peak could be absorbed by kaolin (Table 1) When serum was treated before the chromatography no removal of antibodies could be demonstrated (Fig 2)

On the basis of quantitative estimations of whole immunoglobulin classes most investigators presume that rubella HI antibodies of the IgM class can be absorbed by kaolin No reduction in HI titer of rubella IgM antibody was observed in the serum examined by us (Fig 3) or the serum examined by *Vesikari & Vaheri* (17) In our opinion there is no reason to believe that rubella IgM antibodies are absorbed by kaolin Investigations in this laboratory (unpublished) have shown that some γ globulins such as AB isoagglutinins rheumatoid factor mononucleosis antibodies and others are almost completely removed by treatment with kaolin while antibody titers against viral and bacterial antigens

are not significantly reduced Investigations are in progress to further elucidate these observations.

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Acta path microbiol scand Section B 79, 686–690, 1971

COMPARATIVE ELECTROPHORETIC AND SEROLOGICAL ANALYSES OF *VIBRIO COMMA* AND *AEROMONAS LIQUEFACIENS* PROTEINASES

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The occurrence of one, or two, proteinase fractions was demonstrated for 5 strains of *Vibrio comma* using the zymogram technique for proteinases Most of the proteinase fractions moved more or less rapidly towards the cathode, although some were shown to migrate in the opposite direction at pH 6 2 Serological cross reactions were observed between proteinase fractions produced by a strain of *Aeromonas liquefaciens* and 2 of the *V comma* strains examined The proteinase A of *Ae liquefaciens* was shown to be enzymoserologically identical, or closely related, to one of the proteinase fractions of *V comma*, while no relationship was observed between the proteinase B of *Ae liquefaciens* and any of the *V comma* proteinases In order to remove the naturally occurring proteinase inhibitors from the antiproteinase containing γ globulins in immune serum, precipitation with sodium sulphate was used, with success, as demonstrated by disc electrophoresis and the Casein Precipitation Inhibition test (CPI test) The need for suitable separation techniques when using enzymes as a basis for the taxonomical differentiation of the corresponding organisms is emphasized

A type of partial enzymoserological relationship is described between some strains of *Vibrio comma*, on one hand, and *Aeromonas liquefaciens* and *Pseudomonas aeruginosa* on the other (*Sandvik* & *Dahle* 1971) It was assumed that the relationship was due to at least one of two, or more, enzymes in complexes of proteinases produced by the organisms

A zymogram technique has been developed (*Dahle* 1970) for analysing the complexity of enzymes in mixtures of proteinases By this method, which is based on the electrophoretic separation of the proteinases in agar gel and subsequent development by covering with a sodium caseinate containing gel, the different proteinases can be localized The technique can also be used to demonstrate serologically identical proteinase fractions, in different solutions, by incorporating antiserum against particular fractions in the developing system However, for such purposes the naturally occurring proteinase inhibitors must be removed from the immune sera before addition to the developing system (*Dahle* 1969 a, 1970)

Analyses of the proteinase fractions pro-

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duced by 5 strains of *V comma*, and their serological and electrophoretic relationships to the previously described (*Dahle* 1969 b) *Ae liquefaciens* proteinases, were carried out using the above mentioned technique

MATERIALS AND METHODS

Strains

The strains used were *Aeromonas liquefaciens* (ATCC* 14715), *Vibrio comma* (ATCC 14035), *V comma* (ATCC 14102), *V comma* type Inaba (ATCC 9459), *V comma* biotype eltor (ATCC 14734) and *V comma* biotype eltor (NCTC** 8457)

Enzymes

The proteinases were produced as described previously (*Sandvik* & *Dahle* 1971)

Sera

Specific antiproteinases were produced against the *Aeromonas* and *Vibrio* proteinases as described (*Dahle* 1969 a)

Antienzymes lacking naturally occurring proteinase inhibitors were prepared from the sera by sodium sulphate precipitation of the γ globulins and resuspension of the precipitate in saline to one fifth of the original volume (*Stelos* 1967)

Electrophoresis

Disc electrophoresis, according to *Davis* (1964) was used to test the fractions for the presence of α β, and γ-globulins The separation was carried o t n 7

(7 per cent polyacrylamide), the samples passed through a concentrating gel of 2 5 per cent polyacrylamide The current per tube was adjusted to 1 25 mA through the concentrating gel and to 3 mA through the separating gel Usually 8 to 10 tubes were run simultaneously Bromphenol blue was used as marker substance for the fronts The gels were stained with a 1 per cent solution of amidoblack in 7 per cent acetic acid and differentiated in 7 per cent acetic acid

The Casein Precipitation Inhibition test (CPI test) was used to analyse the sera and fractions for naturally occurring proteinase inhibitors and induced antiproteinases (*Dahle* 1969 b) The CPI-test is based on the separation of the antiproteinases in immune sera from the naturally occurring proteinase inhibitors by paper electrophoresis followed by demonstration of inhibition of the Casein Precipitation reaction (CP-reaction) in the area of the antiproteinases

Zymograms

Zymograms of the proteinases were prepared as described by *Dahle* (1970) The electrophoresis was carried out in 1 per cent agar (Difco*, Special Noble agar, 0142–01) in 0 05 M phosphate buffer pH 6 2 Agar gels were poured onto microscope slides (LKB** –equipment for immunoelectrophoresis) and the electrophoresis run for 60 minutes at 300 V, with 0 05 M phosphate buffer pH 6 2 as electrode buffer After the electrophoresis, the developing agar containing sodium caseinate was poured onto the electrophoresis gel When a particular proteinase was to be neutralized, the developing system also included the corresponding antiproteinase added as purified γ-globulins (*Dahle* 1969 a)

RESULTS

All the strains included in the experiments produced one, or more, proteinase fractions as demonstrated by the zymograms (Table 1) The electrophoretic migration rates were, however, not identical for the proteinases produced by the various strains of *V comma* While most of the proteinase fractions moved more, or less, rapidly towards the cathode, some were seen to migrate in the opposite direction (Fig 1 b) In addition, it was observed that the migration of the proteinases produced by certain *V comma* strains (ATCC 14035, type Inaba ATCC 9459 and biotype eltor ATCC 14734) changed after storing the enzyme containing solution for several days at 4°C

In order to demonstrate serological relationships between proteinases of different origin, the induced antiproteinases in the purified γ globulin fractions were added to the developing system of the zymograms Fig 2 shows the electrophoretic patterns of the

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Acta path microbiol scand Section B 79, 686–690, 1971

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COMPARATIVE ELECTROPHORETIC AND SEROLOGICAL ANALYSES OF *VIBRIO COMMA* AND *AEROMONAS LIQUEFACIENS* PROTEINASES

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The occurrence of one, or two, proteinase fractions was demonstrated for 5 strains of *Vibrio comma* using the zymogram technique for proteinases Most of the proteinase fractions moved more or less rapidly towards the cathode, although some were shown to migrate in the opposite direction at pH 6 2 Serological cross reactions were observed between proteinase fractions produced by a strain of *Aeromonas liquefaciens* and 2 of the *V comma* strains examined The proteinase A of *Ae liquefaciens* was shown to be enzymoserologically identical, or closely related, to one of the proteinase fractions of *V comma*, while no relationship was observed between the proteinase B of *Ae liquefaciens* and any of the *V comma* proteinases In order to remove the naturally occurring proteinase inhibitors from the antiproteinase containing γ globulins in immune serum, precipitation with sodium sulphate was used, with success, as demonstrated by disc electrophoresis and the Casein Precipitation Inhibition test (CPI test) The need for suitable separation techniques when using enzymes as a basis for the taxonomical differentiation of the corresponding organisms is emphasized

A type of partial enzymoserological relationship is described between some strains of *Vibrio comma* on one hand and *Aeromonas liquefaciens* and *Pseudomonas aeruginosa* on the other (*Sandvik & Dahle* 1971) It was assumed that the relationship was due to at least one of two, or more, enzymes in complexes of proteinases produced by the organisms

A zymogram technique has been developed (*Dahle* 1970) for analysing the complexity of enzymes in mixtures of proteinases By this method, which is based on the electrophoretic separation of the proteinases in agar gel and subsequent development by covering with a sodium caseinate containing gel the different proteinases can be localized The technique can also be used to demonstrate serologically identical proteinase fractions, in different solutions, by incorporating antiserum against particular fractions in the developing system However, for such purposes the naturally occurring proteinase inhibitors must be removed from the immune sera before addition to the developing system (*Dahle* 1969 a, *1970*)

Analyses of the proteinase fractions pro

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duced by 5 strains of *V comma*, and their serological and electrophoretic relationships to the previously described (*Dahle* 1969 b) *Ae liquefaciens* proteinases were carried out using the above mentioned technique

MATERIALS AND METHODS

Strains

The strains used were *Aeromonas liquefaciens* (ATCC* 14715), *Vibrio comma* (ATCC 14035) *V comma* (ATCC 14102) *V comma* type Inaba (ATCC 9459), *V comma* biotype eltor (ATCC 14734) and *V comma* biotype eltor (NCTC** 8457)

Enzymes

The proteinases were produced as described previously (*Sandvik & Dahle* 1971)

Sera

Specific antiproteinases were produced against the *Aeromonas* and *Vibrio* proteinases as described (*Dahle* 1969 a)

Antienzymes lacking naturally occurring proteinase inhibitors were prepared from the sera by sodium sulphate precipitation of the γ globulins and resuspension of the precipitate in saline to one fifth of the original volume (*Stelos* 1967)

Electrophoresis

Disc electrophoresis according to *Davis* (1964) was used to test the fractions for the presence of α β and γ globulins The separation was carried out in 7 per cent polyacrylamide gel in glass tubes with 0 05 M Tris HCl buffer pH 8 07 and Tris glycine buffer pH 8 91 as lower and upper buffers respectively Before entering the separating gel (7 per cent polyacrylamide) the samples passed through a concentrating gel of 2 5 per cent polyacrylamide The current per tube was adjusted to 1 25 mA through the concentrating gel and to 3 mA through the separating gel Usually 8 to 10 tubes were run simultaneously Bromphenol blue was used as marker substance for the fronts The gels were stained with a 1 per cent solution of amidoblack in 7 per cent acetic acid and differentiated in 7 per cent acetic acid

The Casein Precipitation Inhibition test (CPI test) was used to analyse the sera and fractions for naturally occurring proteinase inhibitors and induced antiproteinases (*Dahle* 1969 b) The CPI test is based on the separation of the antiproteinases in immune sera from the naturally occurring proteinase inhibitors by paper electrophoresis followed by demonstration of inhibition of the Casein Precipitation reaction (CP reaction) in the area of the antiproteinases

Zymograms

Zymograms of the proteinases were prepared as described by *Dahle* (1970) The electrophoresis was carried out in 1 per cent agar (Difco*, Special Noble-agar, 0142 01) in 0 05 M phosphate buffer, pH 6 2 Agar gels were poured onto microscope slides (LKB** –equipment for immunoelectrophoresis) and the electrophoresis run for 60 minutes at 300 V with 0 05 M phosphate buffer, pH 6 2 as electrode buffer After the electrophoresis the developing agar containing sodium caseinate was poured onto the electrophoresis gel When ...

(*Dahle* 1969 a)

RESULTS

All the strains included in the experiments produced one, or more, proteinase fractions as demonstrated by the zymograms (Table 1) The electrophoretic migration rates were, however, not identical for the proteinases produced by the various strains of *V comma* While most of the proteinase fractions moved more or less rapidly towards the cathode, some were seen to migrate in the opposite direction (Fig 1 b) In addition it was observed that the migration of the proteinases produced by certain *V comma* strains (ATCC 14035 type Inaba ATCC 9459 and biotype eltor ATCC 14734) changed after storing the enzyme containing solution for several days at 4°C

In order to demonstrate serological relationships between proteinases of different origin, the induced antiproteinases in the purified γ globulin fractions were added to the developing system of the zymograms Fig 2 shows the electrophoretic patterns of un

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** LKB Instruments Ltd Stockholm – Bromma Sweden

DISCUSSION

The observations that 4 of the 5 strains of *V comma* examined produced more than one extracellular proteinase fraction supports the assumption that the *V comma* proteinases represent a complex system (*Sandvik* & *Dahle* 1971) In addition, the observed cross neutralizations between the proteinase-antiproteinase systems of *V comma* and *Ae liquefaciens* indicate serological indentity, or close relationship, between particular proteinase fractions of the organisms This is in accordance with the earlier results obtained with the CPI test Serological relationship between one of the two *Ae liquefaciens* proteinases (A+B) and the single proteinase of *Ae salmonicida* (B) has been described previously by *Dahle* (1969 b, 1970) The zymograms in the present work show that the proteinase A (fast moving) of *Ae liquefaciens* is enzymoserologically identical to, or closely related to, one of the proteinase fractions of certain strains of *V comma,* while no relationship was observed between the proteinase B (slow moving) of *Ae liquefaciens* and any of the *V comma* proteinases

The zymogram technique seems to be of value for this type of study, because separation of the individual fractions is carried out before the serological neutralization From a serological point of view, however, it is important to remove the naturally occurring proteinase inhibitors from the immune serum This was effectively obtained by precipitation with sodium sulphate, as demonstrated by the CPI-test, which gave functional evidence for the removal (Fig 3) of the naturally occurring proteinase inhibitors The patterns obtained by disc electrophoresis (Fig 2) indicate that the α and β globulins are excluded, as compared with the patterns for human serum published by *Clarke* (1964)

The different electrophoretic mobilities of the serologically closely related fractions of *V comma* and *Ae liquefaciens* should be seen in connection with the observed differences in the electrophoretic mobilities of one particular proteinase fraction before, and after storage These differences may be caused by the removal from the molecules, of charged groups which do not interfere in the serological reactions, and may be considered a warning against the use of the electrophoretic migration rates of enzymes as the only basis for taxonomical differentiation

Gonzalez (1963) performed experiments with lipases from vibrions and aeromonads and their corresponding antilipases, but, in contrast to the observations with proteinases, he observed no cross reactions between the genera It should be emphasized that the demonstrated cross-neutralizations between the complexes of proteinases and antiproteinases were dependent on individual examinations of each enzyme fraction in question Thus, the need for suitable separation techniques when studying enzymes in relation to taxonomical questions seems to be very important

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DETERMINATION OF BILE ACID CONVERSION POTENCIES OF INTESTINAL BACTERIA BY SCREENING *IN VITRO* AND SUBSEQUENT ESTABLISHMENT IN GERMFREE RATS

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More than 100 facultatively and strictly anaerobic strains from 22 genera isolated from the digestive tract of the rat were tested for the ability to split glycine and taurine conjugates and to transform cholic acid Strains belonging to 14 of these genera, of which 12 were strictly anaerobic, were capable of splitting conjugates, whereas strains from only 7 genera were capable of transforming cholic acid None of the strains isolated could 7α dehydroxylate cholic acid Twenty strains from 9 genera capable of splitting conjugates *in vitro* were established as monocontaminats in ex germfree rats Of these, three strains from the genera *Clostridium, Eubacterium* and *Streptococcus* (strictly anaerobic species) gave approximately 80 per cent splitting of the caecal bile acids Four strains showed no conversion of caecal bile acids, while the remainder showed splitting varying between 4 and 44 per cent of the caecal bile acids There was no correlation between the viable numbers of bacteria in the caecum and the extent of splitting of caecal bile acids Deconjugation *in vitro* cannot be used as a criterion for the potential deconjugation of microorganisms growing in the digestive tract

The microflora in the digestive tract is essential for deconjugation of glycocholic and taurocholic acids and for the important transformation by 7α-dehydroxylation of the primary bile acids, cholic and chenodeoxycholic, into deoxycholic acid and lithocholic acid, respectively This has been demonstrated in comparative studies on conventional and germfree rats (7) Cholic acid is excreted as taurine and glycine conjugates in germfree rats, whereas in conventional rats these acids are deconjugated to 100 per cent and a variety of metabolites are excreted (6) Eighty per cent of the total amount of bile acids excreted have been 7 α-dehydroxylated (6).

A large number of bacterial strains from 15 genera normally present in the intestinal microflora, that have originated from international collections, or have been isolated by various workers, hydrolyzed the conjugated bile acids *in vitro* (1, 14, 15) Some of these strains were able to perform a number of other transformations of bile acids (8,1)

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TABLE 2 *Percentage Conversion of Bile Acids in the Caecum and Colon + Rectum of Monoc*
and, for Comparison, in vi

Animal number	Bacterial strains	Log$_{10}$ number of viable organisms/g of fresh sample		In vitro convers	
				Hydrolysis of**	
		Distal small intestine	Caecum	GDC	TDC
1	*Lactobacillus* 18 × 1 (homofermentative sp)	9 0	9 3	+	+
2	*Lactobacillus* 18 × 6 (heterofermentative sp)	7 3	7 0	+	+
3	*Bifidobacterium* 30 × 11	5 6	8 9	+	+
4	*Eubacterium* 28 × 7	6 0	9 3	+	+
5	*Eubacterium* 28 × 7	2 0	8 5		
6	*Catenabacterium* 28 × 2	6 9	8 6	0	+
7	Isolate from rat 6 caecum	4 5	9 0		
8	*Butyribacterium* 31 × 3	8 0	10 0	+	+
9	*Corynebacterium* 29 × 2 (strictly anaerobic sp)	9 9	10 0	+	+
10	*Streptococcus* 48 × 6 (strictly anaerobic sp)	8 8	9 5	+	+
11	*Streptococcus* 48 × 4 (strictly anaerob c sp)	4 3	5 7	+	+
12	Isolate from rat 11 caecum	4 3	6 5		
13	*Clostridium butyricum* 1010 (SBL)	9 0	9 0	+	+
14	*Clostridium* 34 × 4	7 3	8 3	+	+
15	*Clostridium* 34 × 5	6 5	9 0	0	+
16	*Clostridium* 34 × 11	7 5	8 6	+	+
17	*Clostridium* 34 × 11	4 3	8 0		
18	*Clostridium* 34 × 1	6 3	7 7	0	+
19	*Clostridium* 34 × 7	6 5	8 0	+	+
20	*Acuformis* 24 × 1	5 5	9 0	0	+
21	*Pasteurella* 27 × 2 (strictly anaerobic)	8 0	8 8	+	+
22	*Pasteurella* 27 × 2	7 3	9 6		
23	*Endosporus* 33 × 1	7 3	8 9	+	+
24	*Endosporus* 33 × 2	6 5	8 0	0	+
25	*Endosporus* 33 × 5	7 3	9 3	+	+

§ No analysis performed due to insufficient amounts of labelled bile acids present
* The following abbreviations are used GDC – glycodeoxycholic acid TDC – taurodeoxycholic acid C = cholic acid CD – chenodeoxycholic acid L – lithocholic acid
** + more than 5 per cent of the conjugates hydrolyzed 0 = no detectable hydrolysis
*** More than 5 per cent of the labelled bile acid transformed into metabolites with the thin layer chromatographic behaviour of the following metabolites — Metabolites of *cholic acid* (C) monr

erophorus and *Endosporus* Of the 124 strains tested, 83 were capable of deconjugation and 72 of these deconjugated both glyco and taurodeoxycholic acid The 10 strains that split only taurodeoxycholic acid belong to *Catenabacterium* (2 strains), strictly anaerobic *Streptococcus* species (2 strains) *Clostridium* (3 strains), *Acuformis* (1 strain) and *Endosporus* (2 strains) One strain of *Inflabilis* only deconjugated glycodeoxycholic acid

Transformation of cholic acid was carried out by strains from 8 of the genera examined These strains belong to the genera *Lactobacillus* (homofermentative species) (5 strains), *Eubacterium* (2 strains) *Ramibacterium* (1 strain), *Streptococcus* strictly anaerobic species (2 strains), *Clostridium* (10 strains), *Escherichia* (2 strains) *Sphaerophorus* (1 strain) and *Endosporus* (3 strains) The transformation products of cholic acid had the TLC behaviour of a dihydroxy monoketo

…d Exgermfree Rats with Numbers of Viable Bacteria in the Distal Small Intestine and Caecum …tion of Bile Acids

…e acids*			*In vivo* percentage conversion of bile acids			
			Caecum		Colon + rectum	
Transformation of***						
C	CD	L	Hydrolysis of conjugates	Transformation of unconjugated bile acids	Hydrolysis of conjugates	Transformation of unconjugated bile acids
monoketo	0	(3 keto)	0	0	0	0
0	0	0	0	0	0	0
0	0	0	18	16	34	30
monoketo	0	0	58	20	74	35
			81	10	83	15
0	0	0	16	0	20	0
			15	0	16	0
0	0	0	0	0	0	0
0	0	0	16	0	21	0
monoketo	(7 keto)	(3 keto) (3β OH)	76	25	§	§
monoketo	(3β OH, 7α OH)	(3β OH)	10	0	12	0
			44	0	42	0
monoketo	monoketo	(3 keto)	71	0	67	0
monoketo	(7 keto)	0	20	5	34	4
monoketo	(7 keto)	0	30	20	23	20
monoketo	(7 keto)	0	18	0	19	0
			7	0	5	0
monoketo	(7 keto)	0	7	3	§	§
monoketo	(7 keto)	0	5	2	5	2
0	0	0	4	0	§	§
0	0	0	0	0	0	0
			0	0	0	0
0	0	0	12	0	13	0
monoketo	(7 keto)	0	6	3	6	4
monoketo	(7 keto)	0	18	16	16	14

…keto – dihydroxymonoketo derivatives of cholanoic acid Metabolites of *chenodeoxycholic acid (CD)*
(3β OH 7α OH) – 3β, 7α–dihydroxy–5β–cholanoic acid
(7–keto) – 3α–hydroxy 7–keto–5β–cholanoic acid
(monoketo) – monohydroxymonoketo derivatives of cholanoic acid
Metabolites of *lithocholic acid (L)* (3β OH) – 3β–hydroxy–5β–cholanoic acid
(3–keto) 3 keto–5β–cholanoic acid

derivative of cholic acid Not a single strain of the 124 tested showed the ability to 7 α dehydroxylate cholic acid under the test conditions

In vivo studies Representative strains capable of deconjugation *in vitro* from 9 of the genera known to occur in the dominant flora of the rat (12) were chosen These comprised *Lactobacillus*, *Bifidobacterium Eubacterium*, *Catenabacterium*, *Corynebacterium* (strictly anaerobic species) *Clostridium Endosporus*, *Streptococcus* (strictly anaerobic species) a… *Acuformis* Strains of *Pasteurella* (stric… anaerobic species) were included as an e… ample of the non-sporeforming Gram neg… tive strict anaerobes In all, 28 strains w… selected for establishment studies The p… centage hydrolysis of conjugated bile ac… and transformation of unconjugated b… acids in the caecum, colon + rectum a… faeces were measured after one…

The results obtained

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Acta path microbiol scand Section B 79 699 707 1971

ANTIGEN ANALYSIS ON SERUM FROM CATTLE WITH LEUCOTIC TUMOURS

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This study was performed to examine i[f ch]anges might be found in the serum proteins of cattle with various forms o[f leucosis] [by] immunodiffusion and immunoelectrophore[sis]. An absorption technique in Fourteen serum samples originating from cattle with leucosis had a decrease[d] absorbing antibody against serum from non leucotic animals This is due t[o a de]creased level of a certain protein component. The poss b lity that this pro[tein is] IgM is discussed Loss or change in concentration of this protein seems not to [be specific for] leucos[is] since it was found to be missing also in 4 animals with other d[iseases. Evi]den[c]e of the existence of serum prote ns specif c for leucosis has not been [found].

[In] 1965 a committee was appointed to [establish cri]teria for the determination of the norm[al an]d leucotic state in cattle (International Committee on Bovine Leucosis 1968) In sa[id re]port is mentioned "Thus far a specific vira[l or] cellular bovine leucosis antigen(s) has n[ot be]en demonstrated. Emphasis should [be pla]ced on investigations designed to det[ect] [suc]h an antigen(s) by complement fixati[on], [fluo]rescence inhibition, immunofluorescence and [ot]her immunologic techniques"

Antigenic changes may happen in man[y dif]ferent virus-induced tumours and leu[caemias] (*Old & Boyse 1965*) Some of the antigenic alterations in tumours have been [d]etected by immunodiffusion (*Itakura 1963*, *1964 Takayanagi 1966*) This meth[od has al]so been used for investigation on [leucaemia] in man and mice (*Fink et al 1965*, [*Takayan*]*agi 1965, Fink & Coules 1968*)

[Investigations] on bovine leucosis using immuno[diffusion] and immunoelectrophoresis are rela[tively] f[e]w Antigenic reduction or loss of [norma]l lymph node tissue antigens in bovine [lympho]sarcoma has been found using double [diffusion] (*Yang & Hare 1967*) By means of a[n immuno]electrophoretic technique it has [been] f[ound] that many cows with leucosis do [no]t have IgM in their serum or only a slight am[ount] (*Trainin 1967, 1969 Trainin et al 1969 Klopfer & Trainin 1970*)

Antigenic gain has been demonstrated in bovine lymphosarcoma by *Gillette et al* 1969 [by] immunofluorescence

The present study deals with immunodiffusion experiments on sera from normal cattle and from cattle with leucosis in order to investigate whether any differences might be seen.

Received 22.xi.71

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No	Breed	Age in years	Date of *post mortem* exam	Haematol exam	Path anatomy	Examinations in the herd of origin: Haematol performed	Group*	Classification°
L1	RDM §	ab 5	March 21, 1967	16,700 leucocytes per cmm	Leucosis +, Endometrit postpuerperal purulent	March 19, 1967	I 33 cows II 5 cows III 8 cows	C
L3	RDM	5–6	June 14, 1967		Leucosis 0, Reticulo sarcoma	June 6, 1967 May 29, 1968 Nov 11, 1968 May 20, 1969	I 8 cows I 10 cows I 10 cows I 9 cows	Not leucosis
L5	RDM	4	March 8, 1968	38,900 leucocytes per cmm Diff 63 5 24 5–0 0–12 0–0 0–0 0	Leucosis +, Abomasitis chr erosiv	March 19, 1968	I 21 cows II 1 cow III 9 cows	C
L6	SRB	5–6	May 14, 1968		Leucosis +	May 14, 1968		C
L7	Jersey	Newborn	May 17, 1968	28,900 leucocytes per cmm Diff 0 0–97 0–0 0 3 0–0 0 0 0	Leucosis +	May 21, 1968	I 26 cows	A
L8	SDM	ab 2½	May 25, 1968	16,000 leucocytes per cmm Diff 9 0–71 0–0 0 19 0–1 0 0 0	Leucosis +	June 5, 1968	I 11 cows	A
L9	RDM	elderly	June 7, 1968	14,600 leucocytes per cmm Diff 0 0–45 5–0 0 54 0–0 5–0 0	Leucosis +, Peritonitis *purul et fibr* adhæs chron	July 7, 1968 Aug 28, 1968 The two cows in group III still in group III	I 14 cows II 0 cows III 2 cows	C
L 10	RDM	ab 3½	June 7, 1968	17 600 leucocytes per cmm Diff 35 0–60 0–0 0–5 0–0 0–0 0	Leucosis +, (Lymphndd heart, liver, spleen and	1 leucocytes tumour case in a young bull in Oct 1961 Oct 25, 1961 June 7, 1968	I 29 cows I 37 cows	C

						Aug 19 1969	I 34 cows	
L11	RDM	ab 2	June 15 1968	214 000 leucocytes per cmm	Leucosis +	1 leucotic tumour case in a cow in May, 1968 July 4, 1968	 I 20 cows II 0 cows III 2 cows	C
L12	SDM	ab 2	July 25 1968	5 000 leucocytes per cmm Diff 0 0 55 5 5 5 32 5–4 5 2 0	Skin leucosis + (Lymphndd thymus lungs liver)	Aug 19, 1968 The two cows in groups II and III later on turned out normal	I 28 cows II 1 cow III 1 cow	D
L13	RDM	>2	Aug 28 1968	Group III	Leucosis +	Sept 19, 1968	I 17 cows II 0 cows III 1 cow	C
L14	Jersey	1/12			Leucosis +	Dec 18 1968	I 20 cows	A
L15	RDM	>2	Febr 5 1969		Leucosis 0 Reticulo sarcoma	Jan 25, 1969 Oct 27, 1969 Febr 4, 1970	I 8 cows I 6 cows I 7 cows	Not leucosis
L17	SDM	3/4	June 10, 1969	13 800 leucocytes per cmm Diff 6 0–84 0– 0 0–10 0–0 0–0 0	Leucosis + (All lymphndd)	Apr 29, 1969	I 16 cows	A
L18	RDM	ab 5	Sept 9 1969		Leucosis 0 (Galactopho ritis, mastitis)	Sept 29, 1969	I 10 cows II 0 cows III 0 cows	Not leucosis
L19	SRB	>4	June 15, 1970	3,400 leucocytes per cmm Diff 1 0–80 0– 0 0–18 0 1 0–0 0	Leucosis + (Lymphndd)			C
L20	SRB	3	June 15, 1970	6 000 leucocytes per cmm Diff 0 0–71 0 0 0–26 0–3 0–0 0	Leucosis 0 (Lympha denitis, Reti culoperiton chron traumat)			Not leucosis

Description of individual cases						Examinations in the herd of origin		
No	Breed	Age in years	Date of *post mortem* exam	Haematol exam	Path anatomy	Haematol performed	*Group**	Classification°
L1	RDM §	ab 5	March 21, 1967	16,700 leucocytes per cmm	Leucosis +, *Endometrit* postpuerperal *purulent*	March 19, 1967	I 33 cows II 5 cows III 8 cows	C
L3	RDM	5–6	June 14, 1967		Leucosis 0, Reticulo sarcoma	June 6, 1967 May 29, 1968 Nov 11, 1968 May 20, 1969	I 8 cows I 10 cows I 10 cows I 9 cows	Not leucosis
L5	RDM	4	March 8, 1968	38,900 leucocytes per cmm Diff 63 5 24 5–0 0–12 0–0 0–0 0	Leucosis +, Abomasitis chr erosiv	March 19, 1968	I 21 cows II 1 cow III 9 cows	C
L6	SRB	5 6	May 14, 1968		Leucosis +	May 14 1968		C
L7	Jersey	Newborn	May 17 1968	28,900 leucocytes per cmm Diff 0 0 97 0 0 0–3 0–0 0–0 0	Leucosis +	May 21, 1968	I 26 cows	A
L8	SDM	ab 2½	May 25, 1968	*16 000 leucocytes* per cmm Diff *9 0–71 0–* 0 0 19 0 1 0 0 0	Leucosis +	June 5, *1968*	I *11* cows	A
L9	RDM	elderly	June 7, 1968	14,600 leucocytes per cmm Diff 0 0–45 5 0 0–54 0–0 5–0 0	Leucosis + Peritonitis purul et fibr adhæs chron	July 7, 1968 Aug 28, 1968 The two cows in group III still in group III	I 14 cows II 0 cows III 2 cows	C
L10	RDM	ab 3½	June 7, 1968	17,600 leucocytes per cmm Diff 35 0–60 0–0 0–5 0–0 0 0 0	*Leucosis* +, (Lymphndd, heart, liver, spleen, and	*1 leucocytes tumour case in a* young bull in Oct 1961 Oct 25 1961 June 7, 1968	 I 29 cows I 37 cows	C

						Aug 19 196[illegible]	I 34 cows	
L11	RDM	ab 2	June 15, 1968	214,000 leucocytes per cmm	Leucosis +	1 leucotic tumour case in a cow in May, 1968 July 4, 1968	I 20 cows II 0 cows III 2 cows	C
L12	SDM	ab 2	July 25 1968	5,000 leucocytes per cmm Diff 0 0–55 5–5 5–32 5–4 5–2 0	Skin leucosis +, (Lymphndd, thymus, lungs, liver)	Aug 19, 1968 The two cows in groups II and III later on turned out normal	I 28 cows II 1 cow III 1 cow	D
L13	RDM	>2	Aug 28, 1968	Group III	Leucosis +	Sept 19, 1968	I 17 cows II 0 cows III 1 cow	C
L14	Jersey	1/12			Leucosis +	Dec 18, 1968	I 20 cows	A
L15	RDM	>2	Febr 5, 1969		Leucosis 0, Reticulo sarcoma	Jan 25, 1969 Oct 27, 1969 Febr 4, 1970	I 8 cows I 6 cows I 7 cows	Not leucosis
L17	SDM	3/4	June 10, 1969	13,800 leucocytes per cmm Diff 6 0–84 0–0 0–10 0–0 0–0 0	Leucosis +, (All lymphndd)	Apr 29, 1969	I 16 cows	A
L18	RDM	ab 5	Sept 9, 1969		Leucosis 0, (Galactophoritis, mastitis)	Sept 29, 1969	I 10 cows II 0 cows III 0 cows	Not leucosis
L19	SRB	>4	June 15, 1970	3,400 leucocytes per cmm Diff 1 0–80 0–0 0–18 0–1 0–0 0	Leucosis + (Lymphndd)			C
	SRB	3	June 15, 1970	6 000 leucocytes per cmm Diff 0 0 71 0 0 0–26 0 3 0 0 0	Leucosis 0 (Lymphadenitis Reticuloperitonitis [illegible])			Not leucosis

No	Breed	Age in years	Date of *post mortem* exam	*Haematol exam*	*Path anatomy*	Haematol performed	Group*	Classification°
	Description of individual cases					Examinations in the herd of origin		
L1	RDM §	ab 5	March 21, 1967	16,700 leucocytes per cmm	Leucosis +, Endometrit postpuerperal purulent	March 19, 1967	I 33 cows II 5 cows III 8 cows	C
I 3	RDM	5 6	June 14, 1967		Leucosis 0, Reticulo sarcoma	June 6, 1967 May 29, 1968 Nov 11, 1968 May 20, 1969	I 8 cows I 10 cows I 10 cows I 9 cows	Not leucosis
L5	RDM	4	March 8, 1968	38,900 leucocytes per cmm Diff 63 5 24 5 0 0 12 0–0 0–0 0	Leucosis +, Abomasitis chr erosiv	March 19, 1968	I 21 cows II 1 cow III 9 cows	C
L6	SRB	5–6	May 14, 1968		Leucosis +	May 14, 1968		C
L7	Jersey	Newborn	May 17, 1968	28,900 leucocytes per cmm Diff 0 0–97 0–0 0–3 0–0 0–0 0	Leucosis +	May 21, 1968	I 26 cows	A
L8	SDM	ab 2½	May 25, 1968	16,000 leucocytes per cmm Diff 9 0–71 0–0 0–19 0–1 0–0 0	Leucosis +	June 5, 1968	I 11 cows	A
L9	RDM	elderly	June 7, 1968	14 600 leucocytes per cmm Diff 0 0–45 5 0 0–54 0–0 5–0 0	Leucosis +, Peritonitis purul et fibr adhæs chron	July 7, 1968 Aug 28, 1968 The two cows in group III still in group III	I 14 cows II 0 cows III 2 cows	C
I 10	RDM	ab 3½	June 7 1968	17,600 leucocytes per cmm Diff 35 0–60 0–0 0–5 0–0 0–0 0	I eucosis +, (Lymphndd heart liver, spleen and	1 leucocytes tumour case in a young bull in Oct 1961 Oct 25, 1961 June 7 1968	 I 29 cows I 37 cows	C

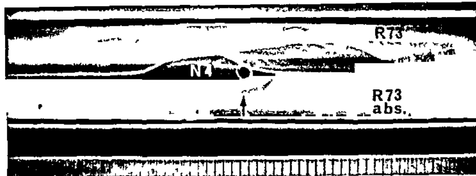


Fig 3 Identification in the total immunoelectrophoretic spectrum of the arc shown in Fig 2 The antiserum is absorbed with L11 Anode to the left

find any specificity in the reaction of this incompletely absorbed antiserum

DISCUSSION

The first point of importance is whether the criteria for nominating the material as normal or pathological are satisfactory Information in this respect has been given previously Another point of interest is to know whether the results are dependent on sex, race, or age

Concerning the sex and race there seems to be no influence As may be seen from Fig 1 a, the antigen which has been found to be missing in the pathological samples may be found in serum from a normal calf, heifer, adult cow, and bull, but not in the five-month old foetus The absorption incapability is found in all pathological sera ranging from a newborn calf to an old cow

It is furthermore important to see if artefacts appear during the long storage periods which were necessary due to scarcity of leucotic tumour cases in Denmark It has been described by *Ritzmann & Levin* (1967) that "pseudo M proteins" may appear in sera from haemolysed blood samples or sera contaminated by bacterial growth Also aged sera may show alteration of the protein pattern In immunoelectrophoresis these various "alien" proteins are placed near the point of application, but such pseudo-M-proteins have not been observed in the samples investigated Loss of an antigen has been demonstrated in serum examined immediately after removing the clot The same pattern was also seen in samples after long term storage at —25°C

It has been described earlier that there is an absence or a decreased level of IgM in sera of leucotic cattle (*Trainin et al* 1968, *Trainin* 1968) In these studies 21 out of 26 cows proven leucotic by histological examination showed absence of IgM in immunoelectrophoresis However, the double diffusion test showed the presence of a small amount of IgM, *i e* in a titre of 1/2–1/4, as compared to 1/24–1/32 in normal cows It has furthermore been found (*Klopfer & Trainin* 1970) that there are fluctuations in the IgM level, so that a leucotic cow with a decreased amount of IgM will on some days have a normal content of this protein component

Considering the above mentioned publications there were obvious reasons to examine if the missing component should be IgM The arc in question is placed near the point of application in the same region as that in which the IgM arc is positioned Its shape and size is, however, not quite as could be expected for an IgM arc Anti bovine IgM which would be ideal for identification is not available at the moment The arc has, as mentioned, not been found in foetal samples This is in agreement with the assumption that it is IgM which is missing in the pathological sera, but it does not prove anything since several components are missing in bovine foetal serum

It is remarkable that the component is

TABLE 1 (Cont.)

No	Breed	Age in years	Date of *post mortem* exam	Haematol exam	Path anatomy	Examinations in the herd of origin: Haematol performed	Examinations in the herd of origin: Group*	Classification°
I 21	RDM	>2	June 17 1970	36,500 leucocytes per cmm Diff 22 0–74 0–0 0–4 0 0 0–0 0	Leucosis +	July 30, 1970	I 17 cows II 0 cows III 2 cows	C
						Sept 11, 1970 The two cows in group III still in group III		

* Groups into which the animals are divided according to the number of lymphocytes per cmm *Bendixen* 1963a, b
° Classification according to the terminology suggested by The International Committee on Bovine Leucosis, Philadelphia, 1965
A Calf, multicentric type
B Thymic type
C Adult multicentric type
D Skin leucosis
§ RDM Danish Red Milk Cattle SRB Swedish Red Milk Cattle SDM Danish Black and White Milk Cattle
† The results of the differential counting are given in the following order (per cent) Large and/or pathological lymphocytes – normal lymphocytes – monocytes – neutrophil granulocytes – acidophil granulocytes – basophil granulocytes



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Strain	O antigen *	Repeating unit §
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SH 2721	*1, 4, 12*	abe glc *O*ac | | –man – rha gal–
{ SH 2204	*1, 9, 12*	tyve glc–*O*ac | man – rha – gal
{ SH 2724		
SH 1360	*6, 7*	–man – man – man – man – g*N*ac–

* The factor 1 depends on the presence of the prophage P22 in all the transductants

§ abe – abequose, man = mannose, rha – rhamnose,
gal – galactose glc = glucose g*N*ac – *N* acetyl glucosamine,
*O*ac – *O* acetyl, tyve = *tyvelose,*

The structures on the first three lines were determined in the strains used in this study (*11*) while the last one is tentative and based on the study of other representatives of the 6 7 antigen (12)

MATERIAL AND METHODS

The bacteria were sister transductants derived from *Salmonella typhimurium* LT2 parent strains, one with the O antigens *4 5, 12,* the other a 5 negative mutant of this (4) The basic unit of the O antigens involved are shown in Table 1 Random bred strain C white mice of both sexes were surgically thymectomized at the age of 7–8 weeks according to Davies, Leuchars Wallis and Koller (5) Ten days after thymectomy the mice were irradiated with a cobalt source The mean dose was 600 rad to the whole body and the immunosuppression did not kill any of the 20 mice that were left un infected

No natural antibodies against any of the studied bacteria could be detected in sera of untreated mice of this strain by passive haemagglutination (Table 2) Mouse erythrocytes were used to exclude the effect of natural hetero agglutinins

The virulence test was started 15–18 hours after irradiation Different dilutions of an overnight broth culture were injected intraperitoneally into groups of ten mice Aliquots were plated for viable counts The LD_{50} values were calculated from 15 day survival data according to the method of *Reed & Muench* (7)

RESULTS AND DISCUSSION

Table 3 shows virulence of the strains possessing different O side-chains in normal and immunosuppressed mice In normal mice the LD_{50} values of the *1, 4, 12* and *1, 4, 5, 12*

TABLE 2 *Passive Haemagglutination Titres*

Sera	Lipopolysaccharide used for coating of cells		
	SH 2201 (*1 4, 5, 12*)	SH 2204 (*1, 9, 12*)	SH 1360 (*6, 7*)
Anti–4, 12 rabbit	6000	100	300
Anti–9, 12 rabbit	1000	6000	300
Anti–6, 7 rabbit	100	100	1000
Nine sera from normal individual strain C mice	all < 1	all < 1	all < 1

The tests were done according to *Beckmann, Luderitz and Westphal* (6)

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Transduction	Strain	O antigen	LD_{50} values at day 15	
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1	SH 2201	1, 4 5, 12	2×10^5	< 50
	SH 2202	1, 4, 5 12	4×10^5	< 50
	SH 2204	1, 9, 12	2×10^6	500
	SH 2205	1, 9, 12	2×10^6	300
2	SH 2721	1, 4, 12	2×10^5	
	SH 2724	1, 9, 12	3×10	
3	SH 1331	1, 4 5 12	2×10^5	<100
	SH 1360	6 7	10^7	10^5
	SH 1361	6 7	10^9	10^6

strains were about 10^5, while the corresponding values for the *1, 9, 12* strains were about 10^6 and for the *6, 7* strains 10^7–10^9 Because preliminary experiments indicated that the immunosuppression drastically decreased the resistance of the mice against *S typhimurium (4, 5, 12)* infection the suppressed mice were infected with smaller inocula than normal mice

The LD_{50} values of these strains in the thymectomized and irradiated mice are also given in Table 3 There was a large difference in virulence between the *1, 4, 5, 12* strain and the *6, 7* strains ($p < 0.001$) Thymectomy and irradiation did not even decrease this difference suggesting that it is not based on differences in antigenicity There was also a statistically significant difference in the virulence between the sister *1, 4, 5, 12* and *1, 9, 12* transductants ($p = 0.01$) The LD_{50} values of the two *1, 4, 5, 12* strains were less than 50, while those of the two *1, 9, 12* strains were 500 to 300 (Table 3) This difference was similar to the difference observed in normal mice

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Other defence mechanisms such as macrophages are less sensitive to these treatments but not insensitive ,Thus neutrophils are moderately irradiation sensitive (9) and the movement of phagocytic cells to the infection site is significantly impaired (10) Even if some of these effects are secondary to the immune suppression it seems obvious that reduction in non specific defence mechanisms as such contributed towards the observed reduction of the average LD_{50} in the thymectomized, irradiated (Tx X ray) mice to *ca* 0 1 per cent of that in the controls

The results above suggest that the differences in virulence between the three sister derivatives of *S typhimurium* are mainly based on a mechanism unrelated to acquired immunity The hierarchy of virulence observed in normal mice (the strains with *1, 4, (5), 12* specific side chains were the most virulent followed first by the strains possessing *1, 9, 12* side chains and finally by strains with *6, 7* side chains) remained essentially unaltered in Tx X ray mice although the LD_{50} values were lower than those in normal mice

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SH 2204 SH 2724	*1,9,12*	tyve glc *Oac* | man rha – gal–
SH 1360	*6,7*	man – man – man – man – gNac–

* The factor 1 depends on the presence of the prophage P22 in all the transductants

§ abe = abequose, man = mannose, rha = rhamnose,
gal = galactose, glc = glucose, g*N*ac = *N* acetyl glucosamine,
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Anti 9,12 rabbit	1000	6000	300
Anti–6,7 rabbit	100	100	1000
Nine sera from normal individual strain C mice	all < 1	all < 1	all < 1

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	SH 2204	1, 9, 12	2×10^6	500
	SH 2205	1, 9, 12	2×10^6	300
2	SH 2721	1, 4, 12	2×10^5	
	SH 2724	1 9, 12	3×10^6	
3	SH 1331	1, 4, 5, 12	2×10^5	<100
	SH 1360	6 7	10^7	10^3
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strains were about 10^5, while the corresponding values for the *1, 9, 12* strains were about 10^6 and for the 6, 7 strains 10^7–10^9 Because preliminary experiments indicated that the immunosuppression drastically decreased the resistance of the mice against *S typhimurium (4 5, 12)* infection the suppressed mice were infected with smaller inocula than normal mice

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SH 2204 / SH 2724	*1, 9, 12*	tyve glc–*O*ac / man rha – gal–
SH 1360	*6, 7*	man – man – man – man – g*N*ac–

* The factor 1 depends on the presence of the prophage P22 in all the transductants

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chains The sugar composition of the *6, 7* antigen differs greatly from that of the other antigens (Table 1) Phagocytic cells killed the *6, 7* strain SH 1360 more rapidly than the sister *1, 4, 5, 12* strains *in vitro* (*Valtonen* & *Ruutu*, to be published)

It is much more difficult to see how mechanisms other than acquired immunity could be responsible for the observed difference of virulence between the sister transductants with the very similar *1, 4, (5), 12* and *1, 9, 12* side-chains Incompleteness of O antigen synthesis resulting in partial or total „roughness" of the bacteria is known to reduce virulence All the strains studied were "smooth" according to cultural and serological reactions and phage sensitivity (1) The lipopolysaccharide of the transductants was analyzed in regard of the amount and arrangement of O specific material the *1, 4, (5), 12* and *1, 9, 12* strains were exactly similar in these respects (11)

A possibility is that the *O*-acetyl groups of antigen *5* might render bacteria less vulnerable to non-specific defence mechanisms than the acetyl group in the glucose position (Table 1) However this seems unlikely as the *1, 4, 12* strain with *O* acetyl in the glucose position was as virulent as *1 4, 5, 12* (Table 3)

Another possible explanation is that the immunosuppression was not complete Its plausibility is diminished by the fact that the immunosuppression did not even reduce the ·ˈˈerence between LD_{50} values although the ˈ olute values went down We are left with ;in explanation which again is not entirely easy to believe that non specific defence mechanisms exhibit a considerable degree of discrimination

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This work was supported by the *Sigrid Juselius Foundation* The expert technical assistance of Mrs *Liisa Pirinen* is gratefully acknowledged

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Acta path microbiol scand Section B 79, 719-720, 1971

BRIEF REPORT

IMMUNOLOGICAL STUDIES ON THE L FORM OF AN *E COLI* STRAIN

Preliminary Report

B Kaijser, J E Brorson, L Å Hanson and *S Seeberg*

The finding of L forms of gram negative bacteria in some patients with urinary tract infection (UTI) has suggested the possibility that such organisms may be the cause of infections in patients where no bacteria can be cultivated from the urine using ordinary media (i a 1, 2) As a basis for studies of the possible role of *E coli* L forms in infections, especially UTI, we wanted to investigate the immunological characteristics of the L form of an *E coli* strain (G 3404/41, serotype O8 K8 H4 from the *WHO International Escherichia Centre*, Statens Seruminstitut, Copenhagen)

Received 16 viii 71 from the *Departments of Clinical Bacteriology and Immunology, Institute of Medical Microbiology, University of Goteborg Goteborg, Sweden*

The L forms were induced by penicillin and repeatedly subcultivated on a penicillin containing L form medium which was a modification of an optimal growth medium for *E coli* in shake culture described by *Holme et al* (3) specially adjusted for L form cultivation This osmotically stabilized, solid medium showed surface growth of microorganisms that fulfilled the following criteria of L forms (4) Growth with the type of colonies, 'L colonies', first described by *Klieneberger Nobel* (5) in contrast to ordinary bacterial growth on solid media Repeated subcultivation was possible No growth of L forms on ordinary media Ethanol fixed, gram stained smears from the colonies contained rounded, gram negative, poorly stained, spherical or pleomorphic bodies but no rods After at least four serial transfers on peni

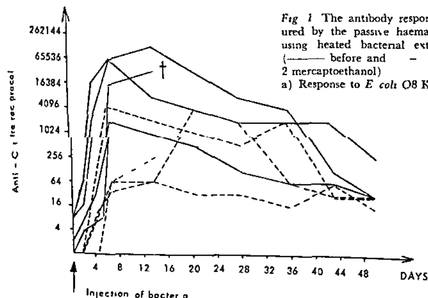


Fig 1 The antibody response in rabbits as measured by the passive haemagglutination technique using heated bacterial extract as antigen (8) (——— before and – – after treatment with 2 mercaptoethanol)
a) Response to *E coli* O8 K8 H4

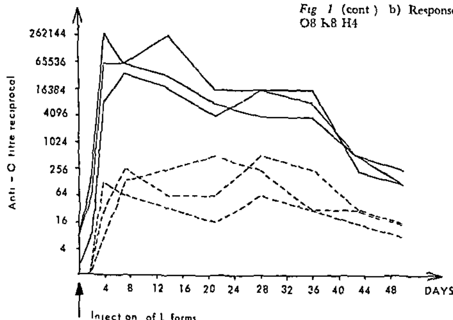


Fig 1 (cont) b) Response to L form of *E coli* O8 K8 H4

cillin containing plates the L colonies were harvested and used for immunization and antigen preparation The immunization schedule and antigen preparation method were the same as for the complete bacteria (6) Immunodiffusion studies of these antigens using antisera from the immunized rabbits showed that the L forms had at least four antigens in common with the complete bacteria They also contained O antigen as indicated by immunodiffusion (7), immunofluorescence and passive haemagglutination techniques (8) but much less than the bacteria as tested by *the single radial immunodiffusion method* (9) There was enough O antigen, however, to induce an O antibody response similar to that of the bacteria on immunization of rabbits (Fig 1) These preliminary observations may be relevant for the studies of the antibody response to O antigen obtained in patients with UTI, since they indicate that an infection caused by L forms may induce an O antibody response This could explain the increased antibody titres which have been noted in a few patients with clinical signs of UTI, but with no positive bacterial cultivation from the urine using ordinary media (10)

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Professor KAI ADOLF JENSEN, M. D.

JULY 16 1894—MAY 2 1971

Professor K A Jensen, for many years editor of Acta Pathologica et Microbiologica Scandinavica, died on May 2, 1971

K A Jensen was born in Copenhagen in 1894 He graduated in medicine from the University of Copenhagen in 1922 and in the same year he joined the scientific staff at Statens Seruminstitut His thesis for the doctor s degree—an interesting and stimulating experimental work on the normal growth of the colon bacillus—was published in 1927

At that time however, he was already deeply engaged in experimental work on the cultivation of tubercle bacilli and soon all his interest concentrated on bacteriology, pathology and epidemiology of tuberculosis In 1931 his laboratory was established as a separate Department of Tuberculosis of which he was appointed

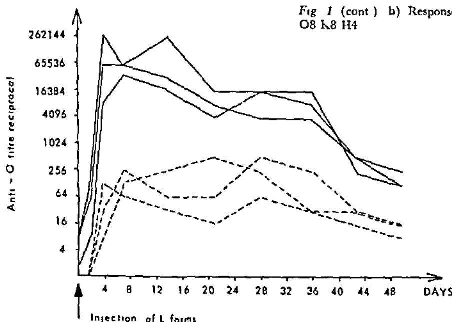


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chief He was professor in general pathology at the University of Copenhagen and director of the Institute of General Pathology from 1940, he retired in 1965 at the age of 70

K A Jensen published alone or together with coworkers—both clinicians and bacteriologists—an imposing number of papers covering practically all aspects of the bacteriology of tuberculosis His department at Statens Seruminstitut was one of the leading tuberculosis laboratories in the world and a centre for research of great importance for the exact diagnosis and for the elucidation of the epidemiology of the disease

At the university he continued the research on tuberculosis soon concentrating on problems concerning the use of antituberculous drugs His studies in this field carried out partly in collaboration with specialists from tuberculosis hospitals, became of the greatest importance At an early time he worked out exact methods for sensitivity determination of tubercle bacilli and advocated a combined treatment in order to avoid as far as possible the development of drug resistance It is, undoubtedly the merit of K A Jensen that the incidence of drug resistant cases of tuberculosis is lower in Denmark than in most other countries

During the occupation of Denmark in World War II K A Jensen succeeded, in cooperation with the scientific staff of Leo Pharmaceutical Products, in producing a preparation of penicillin which—although available only in relatively small quantities—became of great importance for the treatment of cases of severe infections After this imposing achievement he continued investigations on penicillin and later on other antibiotics Just as in the field of tuberculosis, his studies on various antibiotics were of great practical importance for the introduction of the new drugs in Denmark

K A Jensen was a hard working scientist and an inspiring advisor for collaborators and pupils

For almost 25 years K A Jensen was a highly respected member of the Editorial Board of Acta Pathologica et Microbiologica Scandinavica, serving as its Editor in chief from 1962–65 During this period the journal steadily made progress and attained international recognition

He was *doctor honoris causa* at the Royal Veterinary and Agricultural High School in Copenhagen and member of the Royal Danish Academy of Sciences and Letters, as well as honorary member of several scientific societies in Denmark and in other countries

K Riewerts Eriksen

Acta path microbiol Scand Section B 79 721 725 1971

HEPATITIS ASSOCIATED ANTIGEN: ELIMINATION FROM A DIALYSIS UNIT AND PERSISTENCE IN RENAL TRANSPLANT RECIPIENTS

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Testing for hepatitis associated antigen (HAA) in a dialysis unit with previous outbreak of viral hepatitis and similar testing of renal transplant recipients revealed that HAA had been eliminated from the kidney unit by transplantation by chance, of the infected patien s and that transplantation did not lead to any increase in the severity of the disease HAA positive renal transplant recipients remained carriers of HAA with an unusual high titre It is concluded that the prophylactic measures had limited and finally prevented spreading of HAA in the haemodialysis unit

Viral hepatitis is a well recognized hazard to patients and staff in maintenance haemodialysis units Testing for Australia antigen or hepatitis associated antigen (HAA) in kidney units with outbreak of hepatitis (3 4 6, 10) have shown good agreement With a few exceptions all patients on maintenance haemodialysis contracting viral hepatitis have shown positive reactions In contrast to staff members, dialysis patients tend to remain antigen positive indefinitely (6 10) Once infected, eradication of HAA from dialysis units is considered to be extremely difficult

From August 1968 to June 1969 an outbreak of viral hepatitis including staff members had occurred in our haemodialysis unit Testing for HAA was carried out from November 1969 The results of this investigation are reported here

MATERIALS AND METHODS

The Kidney Unit

The dialysis unit at the Rigshospital in Copenhagen came into operation in May 1955 Maintenance haemodialysis had been carried out since 1964 and from January 1968 renal transplantation had been performed From 1964 a single passage system through Kiil dialysers was used, in 1969 succeeded by disposable dialysers (Gambro) The number of patients receiving regular haemodialysis was gradually increased and on January 1st 1969 eleven patiens were treated Access to the blood stream was obtained by means of external arteriovenous shunts or internal arterio venous fistulae

The Outbreak of Hepatitis

Liver function tests had al extensively Since April 1968 pyruvic transaminase (S GPT

Received 25 v 71

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K Rieuwerts Eriksen

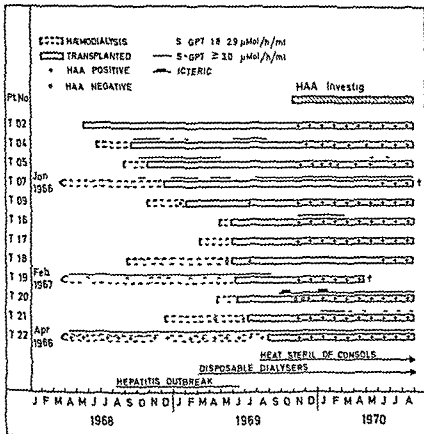


Fig 1 The period of maintenance haemodialysis and time of renal transplantation for the twelve patients whose sera contained hepatitis associated antigen (HAA) From four patients, stored sera were available

HAA could be demonstrated in sera from twelve of the 44 patients The titres of HAA were very high, higher than in any other group of patients yet studied (9) and re mained positive with unaltered high concen trations in all patients

Some data concerning the HAA positive patients are shown in Figure 1 They had all undergone renal transplantation before Sep tember 1969 The series was divided accord ing to this date with a view to disclosing a possible correlation between the presence of HAA and the duration of maintenance haemodialysis or the number of transfusions received As a consequence of the increasing activity of transplantation the du tion of haemodialysis was generally lone r n the group of patients transplanted b r Sep tember 1969 and the number of l d units received correspondingly greater, however, any correlation did not exist (Fig 2)

Only one of the HAA positive transplant patients (T 2) had never been haemodialysed The other eleven HAA positive patients had all undergone *maintenance haemodialysis at* the Rigshospital during the period of out break of hepatitis From four of the HAA positive patients serum samples obtained during their period on dialysis were available and three of them were HAA positive It appears from Figure 1 that the dialysis unit was contaminated with HAA throughout the period of hepatitis outbreak

The liver affection in the *transplant pa* tients who were HAA positive was *characteri* zed by slightly elevated transaminase *levels of* long duration **Only** icteric *Four of* the HAA **positive** d never

weekly in haemodialysis patients and at monthly intervals in the staff members in the kidney unit Renal transplant recipients were tested at their ordinary control visits

Since April 1968 several patients developed moderately elevated transaminase values for long periods In August 1968 and February 1969 two dialysis patients died from hepatitis In May 1969 a former dialysis patient who had received a renal graft in January died in hepatic coma In September 1968 and in January 1969 two dialysis nurses, and in June 1969 two medical officers, developed hepatitis No secondary cases were recorded

Prevention of Infection

The general preventive measures were unaltered during the period under consideration Masks and sterile disposable gowns and gloves were extensively used Syringes, needles and blood lines were disposable Spilled blood was wiped up immediately but disinfectants were not used It was only to a small extent possible to assign hepatitis patients their own dialyser and the consols were virtually used at random

After the onset of the outbreak of hepatitis some important improvements in the general infection preventive measures were introduced From the autumn of 1968 consols and Kiil dialysers were sterilized with 2 per cent formaldehyde In March 1969 Kiil dialysers were replaced by disposable dialysers From August 1969 the consols were sterilized with formalin as well as by heath (80° C) and conventional measuring of pressure in the blood lines by means of tubes connected to a manometer was replaced by disposable blood pressure transducers

γ globulin was only administered when staff members pricked their fingers with needles contaminated with blood from patients with signs of liver affection

Determination of HAA and anti HAA

HAA and anti HAA were detected by precipitin reaction in gel electrophoresis (9) HAA positive sera were quantitated by electrophoresis in antibody-containing agar gel a m Laurell (5)

Screening for HAA and anti HAA

From November 1969 to March 1970 the dialysis patients and all patients in the ward were tested weekly Discharged renal transplant recipients were tested at their ordinary control visits Staff members in the dialysis unit as well as other members of the personnel were tested monthly Serum samples from all individuals were tested for anti HAA During this fourmonth period the possible sources of infection were carefully studied including the number of blood transfusions From March until September 1970 the dialysis patients and the renal transplant recipients were followed with same frequency

Subsequently the intervals between HAA screenings were extended the dialysis patients are now being tested monthly

Patients

The dialysis series comprised twenty-one patients on maintenance haemodialysis, eight of these had undergone dialysis during the period during which the clinical outbreak of hepatitis occured Thirteen patients underwent a short run of haemodialysis

From January 1968 to March 1970 45 patients had undergone renal transplantation One of these died in hepatic coma before our study and serum samples were not available Among the 44 patients who were investigated, twenty had undergone maintenance haemodialysis during the outbreak of hepatitis Two died of other causes during the study, both were HAA negative Twelve of the 44 patients underwent renal transplantation during the study, four of these had undergone maintenance haemodialysis at the Rigshospital and are included in the haemodialysis series too After transplantation all patients received immunosuppressive treatment with prednisone and azathioprine

RESULTS

Dialysis Patients

HAA could not be detected in any of the numerous serum samples from these patients However, serum from one patient contained anti HAA This patient had been haemodialysed over a period of five years After kidney transplantation the antibody disappeared within three months

In May 1970 a patient who was HAA positive prior to admission underwent six haemodialyses before renal transplantation No special precautions were taken in the kidney unit In spite of this contamination the other dialysis patients remained HAA negative The patient remained carrier after transplantation

Renal Transplant Recipients

The number of tests for HAA after transplantation varied from three to 29, averaging fourteen

ducers seemed to have broken the final link in the chain of infection The unit was contaminated for one month after this advent without further spreading of the antigen and recontaminated during May 1970, still without occurrence of secondary cases

The persistence of HAA in very high concentrations in serum from the renal transplant recipients must be attributed to the immunosuppressive treatment since comparatively high titres have only been observed in patients receiving treatment with corticosteroids for other diseases (5) Like dialysis patients (4, 6) signs of liver affection in the transplant patients who were carriers of HAA were either slight or absent, only one became jaundiced (T 20)

Even though spreading of HAA in a dialysis unit may be prevented it is desirable to have carriers of HAA isolated *Knight et al* (3) obtained this by establishing home dialyses In our unit it was retrospectively found that elimination of HAA was obtained by chance by transplantation of the infected patients and that transplantation in these cases, even in the presence of elevated transaminase values, did not seem to imply any increased operative risk or a deterioration of the disease

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Date of Kidney Transplantation

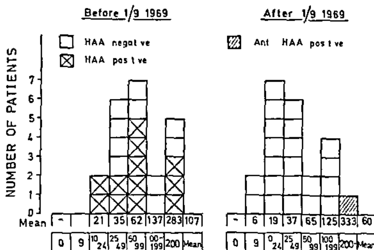


Fig 2 The number of blood transfusions received in patients transplanted before and after September 1969

sented symptoms or signs of hepatitis On the day of transplantation three of the patients had slightly elevated transaminase values which did not increase after operation

The Dialysis Personnel

This group comprised 24 persons including two of the staff members, who had suffered from hepatitis prior to the investigation HAA and anti HAA could not be demonstrated in any of the serum samples and all S GPT values were normal

The Wards

Forty seven patiens and 20 members of the personnel were screened There were no cases of hepatitis and all were HAA negative

DISCUSSION

The incidence of hepatitis in haemodialysis units continues to increase although the risk of infection to which both staff and patients are exposed is fully recognized (1 2) However, none of the eight Danish haemodialysis centres except our unit has experienced an outbreak of viral hepatitis (8) In the present study, the available facts suggest that the eleven HAA positive patients were infected during haemodialyses Three of the patients were known to have been positive at that time and four others had transaminase elevation at the time of transplantation None of the patients transferred for transplantation from other centres were HAA positive After transplantation the patients had not received transfusions and there was no correlation between the number of transfusions previously received and the presence of HAA

HAA or anti HAA could only be detected in twelve of the 27 patients tested who had undergone maintenance haemodialysis during the period in which the kidney unit was contaminated Compared with the much higher incidence reported from other centres (6), this suggests that the preventive measures already from the beginning of the outbreak had limited the spreading of the agent The change to disposable dialysers must be considered as an important advance in the efforts to reduce the general risk of infection (7) However, three of the HAA positive patients started on haemodialysis after this advent In our unit, the introduction of heat sterilization of the consols still using formalin, and the simultaneously change to disposable blood pressure trans

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Acta path microbiol Scand Section B 79, 726–738, 1971

THE PURIFICATION AND SOME PROPERTIES OF TWO *AEROMONAS* PROTEINASES

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Aeromonas liquefaciens and *Aeromonas salmonicida* were both found to produce considerable amounts of extracellular proteinases, when grown on semi solid skim milk agar at 30° C The proteinase A, produced by *Ae liquefaciens* only, and the proteinase B, produced also by *Ae salmonicida* were purified approximately 100 times by precipitation twice with $(NH_4)_2SO_4$, batchwise treatment with DEAE cellulose and, finally, gel filtration on Sephadex G 100 The pH optimum was estimated to be 7 9 for proteinase A and 9 0 for proteinase B Differences between the proteinases were also found with regard to thermoresistance, and to their behaviour in the presence of some naturally occurring proteinase inhibitors The addition of iron and cobalt ions increased the activity of both proteinases Molecular weights were estimated to be 22 100 for the proteinase A and 43 600 for proteinase B on the basis of gel filtration on Sephadex G 100

The serological and electrophoretic behaviour of the extracellular proteinases produced by *Aeromonas liquefaciens* and *Ae salmonicida*, and their corresponding antiproteinases, is described by *Dahle* (1969b) It was shown by means of so called enzymoserological separation that *Ae liquefaciens*, in all media examined, produced a proteinase B serologically identical with, or closely related to, the single proteinase produced by *Ae salmonicida* In addition, a proteinase A, specific for *Ae liquefaciens*, was produced when the organism was grown in certain complex media In view of the use of the bacterial proteinases in the taxonomical differentiation of the organisms in question (*Dahle* 1970, *Sandvik* & *Dahle* 1971), and the variation of different enzyme fractions under various environmental conditions, the analysis and characterization of this kind of enzyme complexes are of importance It is necessary, however, to carry out such investigations on purified materials

The present paper deals with purification of the two *Aeromonas* proteinases, and comparison of some of their biochemical and biophysical properties

MATERIALS AND METHODS

Strains and Cultures

The strains used were *Aeromonas liquefaciens* (ATCC 14715) and *Aeromonas salmonicida* (ATCC 14174) obtained from the American Type Culture Collection (ATCC) Rockville, Maryland U S A For production of proteinases cultures were grown on semi solid skim milk agar (nu

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trient broth (Difco* 0003-01), 40 per cent, nutrient agar (Difco 0069-01), 42 per cent, autoclaved skim milk, 18 per cent) in Roux bottles for 3-4 days at 30° C. Harvesting of the liquid containing the enzymes was performed by freezing the cultures at —20° C, followed by thawing and filtration

Enzymes, Substrates and Inhibitors

The proteinases were identified by the enzymoserological Casein Precipitation Inhibition test (CPI test) The CPI test is based on the separation of the antiproteinases in immune sera from normal proteinase inhibitors by paper electrophoresis, followed by demonstration of inhibition of the Casein Precipitation reaction (see below) in the area of the antiproteinases (*Dahle* 1969b)

Sodium caseinate was obtained from Eastman Kodak Rochester N Y, U S A, and Casein (Hammarsten quality) from Merck, Darmstadt Germany

Serum containing naturally occurring inhibitors against bacterial proteinases was sucked off from blood of various animals after coagulation while inhibitors from other animal and vegetable sources were isolated in saline extracts of the materials (*Fossum* 1970) Proteinase inhibitors isolated from soy bean lima bean chicken egg white and pig pancreas were obtained from Sigma, St Louis Mo U S A

Estimation of Enzymes, Inhibitors and Total Protein

The concentration of proteinases (CP titre) was determined by the Casein Precipitating method of *Sandvik* (1962), as modified by *Dahle* (1969a) A hot solution containing 1 40 per cent Bacto agar 0140-01 from Difco, 1 00 per cent sodium caseinate from Eastman Kodak, (added as a 4 per cent solution pH 6 2), 0 01 per cent merthiolate and 0 004 M $MgCl_2$ in distilled water was poured into a glass tray to a depth of 2 mm and allowed to solidify Aliquots of 25 µl from serial 2 fold dilution of the proteinase solutions were transferred to wells of 3 mm diameter in the agar containing sodium caseinate before incubation at 37° C for 16 hours The estimation of diffusion units was based on the diameters of the precipitation zones which occurred For extremely concentrated proteinase solutions preliminary dilutions were performed before making the serial 2 fold dilutions

Proteolytic activity was determined by a modified *Kunitz* (1947) procedure The digestion of 1 ml 2 per cent casein solution (Hammarsten quality) with 1 ml enzyme solution was performed by incubating the mixtures for 20 minutes at 37° C after which the addition of 3 ml 5 per cent

* Difco Laboratories Inc Michigan, U S A

trichloracetic and [illegible] tivity and precipitated [illegible] The absorbance at 2[illegible] supernatants after centr[illegible] determinations of pH [illegible] buffer pH 8 1 was used [illegible] ases For the determinat[illegible] lowing buffers were used [illegible] 6 0), phosphate buffer (pH 5[illegible] buffer (pH 6 5-9 5) and [illegible] (pH 9 2-12 0)

The effects of the [illegible] inhibitors were tested on [illegible] plates by the crossway [illegible] *Fossum* (1970)

The protein concentration of [illegible] preparations was determined by [illegible] method of *Lowry et al* (1951) [illegible] albumin as the standard

Purification of the Proteinases

The presented sequence of the [illegible] fication procedure (Table 2 and 3) [illegible] several sequences carried out The [illegible] were precipitated with ammon[illegible] per cent saturation at room temper[illegible] lowed to stand for one hour before centr[illegible] at 20 000 g for 20 minutes followed by d[illegible] of the precipitate in small volumes of wa[illegible] subsequent dialysis against tap water [illegible] and finally distilled water at 4° C Before [illegible] the enzyme solutions to a batch of DEAE[illegible] (4° C), the ion exchange material was [illegible] with pure distilled water The elution was [illegible] formed in a Buchner funnel by adding [illegible] volumes of 0 05 M acetate buffer (pH 5[illegible] second precipitation step with ammon[illegible] to 70 per cent saturation followed by d[illegible] water and dialysis against distilled water the [illegible] zymes (3 ml material applied) were eluted fro[illegible] Sephadex G 100 column (1 5 cm / 83 [illegible] (4° C) with the acetate buffer and 3 ml fra[illegible] were collected The CP reaction was used [illegible] detection of the proteinases after the gel fi[illegible] and also in addition to the Kunitz method [illegible] determinations of the activities of the enzym[illegible]

Biophysical Procedures

The material to be heated was transferr[illegible] aliquots of 0 1 ml to thin walled 1 ml ampules After sealing the ampules were [illegible] an ice bath at 0° C until heating wh[illegible] carried out in a Haake (Haake Berlin, G[illegible] ultrathermostate During heating, the [illegible] were completely submerged in the water b[illegible] which they were rapidly cooled in an [illegible] (*Sandvik* 1962) The activities were de[illegible] by the CP method and the method of

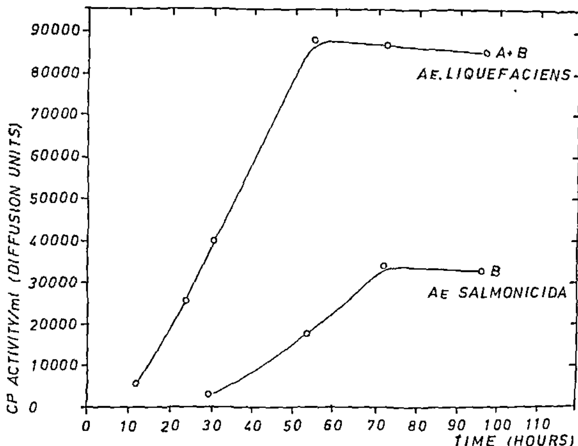


Fig 1 The CP-activities, as a function of time, for cultures of *Aeromonas liquefaciens* and *Aeromonas salmonicida* grown on semi-solid skim milk agar at 30° C, as measured by the CP method The incubation was performed as stationary cultures in Roux bottles At the end of each incubation the bottles were shaken for homogenization before samples of one ml were taken for titration of the CP-activity

The spectrophotometrical measurements were carried out in a Beckman DB spectrophotometer, (Beckman Instruments Inc, Fullerton, California, U S A) and pH values were determined by a pH-meter (Radiometer Copenhagen, Denmark) Disc electrophoresis was carried out in 7 per cent polyacrylamid gel in glass tubes, with 0 05 M Tris HCl buffer, pH 8 07, and glycine NaOH buffer, pH 8 91 as lower and upper buffers respectively (*Dahle & Sandvik* 1971) Immunoelectrophoresis was carried out following standard procedures (*Dahle* 1971)

Chemicals

DEAE-cellulose was obtained from Eastman Kodak, Sephadex G-100 and Blue dextran from Pharmacia, Uppsala, Sweden, while bovine serum albumin, ovalbumin, bovine α-chymotrypsin, pepsin and cytochrome C were obtained from Sigma Other chemicals were of analytical grade, and obtained from Merck

RESULTS

Production of Proteinases

The amounts of proteinases produced by *Ae liquefaciens* and *Ae salmonicida*, as a function of time are shown in Fig 1 for cultures grown on semi-solid skim milk agar at 30° C In all experiments performed, the amount produced by *Ae liquefaciens* was 2 to 3 times greater than that produced by *Ae salmonicida* as measured by the CP-method Whereas the CP-titre was positive for the former strain after 12 hours incubation, the latter needed nearly 30 hours to reach the same level Harvesting of the cultures was carried out after 60 to 80 hours of incubation Table 1 gives the parameters for the proteinase solutions obtained The

. *Comparison of the Proteinase Solutions Obtained by Growing Aeromonas liquefaciens and Aeromonas salmonicida on Semi Solid Skim Milk Agar for 72 Hours at 30° C*

nase ion	Activity per ml CP*	Activity per ml K§	Total protein (mg/ml)	Specific activity CP/mg	Specific activity K/mg
aciens† (A B)	87400	24 90 10 ^	5 7	15320	4 35 10 ²
n cida)	29000	6.34 10⁻²	10 4	2790	0 61 10 ²

usion units determined by the CP method
s determined by the method of Kunit
proteinase solutions are indicated by the ore which the proteinase A and B are pro-
d.

efaciens* solution contained two sero- different proteinases (A and B) and monicida solution only one proteinase shown by the CPI test

tion of the Two Aeromonas *oteinases*

proteinase A was purified from th efaciens solution and B from th monicida solution by equivalent pro s Initial precipitation with ammoniu e (80 per cent saturation) and di ainst running tap water and distil were followed by column chromat on DEAE-cellulose (Fig 2) How n the final procedure, which is pr in Table 2 and Table 3 the colur tographs was replaced by batchw ment with DEAE-cellulose The enzym then eluted from DEAE-cellulose in ner funnel by adding small volum M buffered NaCl solution. Most of th mases could be recovered from the ... material by such elution as can be from Fig 2. This large scale step wa ... because of its unlimited capacity ... the proteinase solutions were ... After a second precipitation ... ammonium sulphate (70 per cent ... which concentrated the solutions ... 3 ml of the enzyme-containing ... were separated further on Sephadex

* The ... was named as the ... and the proteinases

The B enzyme produced by *Ae s* was eluted in a symmetrical peak maximum in fraction number ... A enzyme produced by *Ae liqu* separate experiments was collect fractions under the knee (fractio vn in Fig 3 As no absorban was observed for these eluates ... activity, the curves are bas ... ion (CP titre) only The fra main peak of the *Ae liqu* contained both the enzym own by the CPI test wh nder the knee contained on

ification procedure, the C d for titration of the C purification steps and f the proteinases in the eluat ographs columns while th d for the identification

nzymes showed only on rylamide gel electrophores ... ase A always moved slight ... towards the anode than the proteina B In immunoelectrophoresis the purifi proteinases caused one single precipitatio line while the crude solutions caused nume ous lines D ... 1971 Figs 4a and 4b sho the ultraviolet absorption spectra obtained ... 0 05 M acetate buffer pH 5.6 for the enzym proteins Maxima were observed at 277 n and 2?? nm for the proteinase A and B r spectively

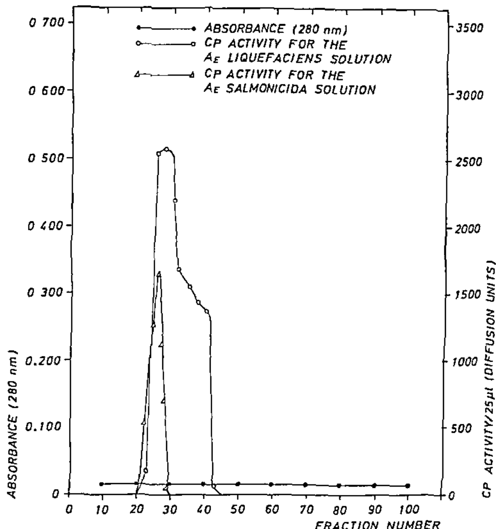


Fig 3 Gel filtration on Sephadex G-100 of the proteinase A and B after the fourth purification step The column (1 5 × 80 cm) was in both experiments equilibrated with 0 05 M acetate buffer, pH 5 6, and 3 ml sample applied Elution was performed with the acetate buffer and the eluate collected as 3 ml fractions The CP reaction was used for the demonstration of the proteinases after the column *chromatography and the absorbance at 280 nm was read in a Beckman DB spectrophotometer*

to 34 times when added to final concentrations of 0 5 10 ³ M Subsequent addition of EDTA to 0 5 10 ³ M reduces the effect of cobalt, but raises the activities further to 60 to 70 times the original for the solutions which were treated with iron When EDTA alone is added to the enzyme solutions, the activity of proteinase A is halved, while the effect on proteinase B is negligible

Effect of naturally occurring proteinase inhibitors The naturally occurring proteinase inhibitors, which are widely distributed in nature (*Fossum* 1970), are not known to be very specific in their inhibition of bacterial proteinases As *Ae liquefaciens,* under certain circumstances, is able to produce both the proteinases A and B it was of interest to compare the enzymes against some naturally occurring proteinase inhibitors of animal and vegetable origin Fig 8 shows the white precipitation of sodium caseinate, in agar gel caused by the proteinases, and the interruptions in the precipitation caused by some inhibitors Table 5 summarizes the results

TABLE 3 *Purification of the Proteinase B Produced by* Aeromonas salmonicida

Purification step	Volume (ml)	Total protein (mg)	Activity CP* 10^{5}	K§	Specific activity CP/mg	K/mg	Recovery (%) CP	K	Fold purification CP	K
I Centrifuged culture filtrate	800	8320	232	51	2780	0 006	100	100	1	1
II 80 % (NH_4) SO_4 precipitate (after dialysis)	130	464	181	37	39000	0 080	78	72	14	13
III Batchwise treatment with DEAE cellulose	210	105	84	19	80000	0 181	36	37	29	30
IV 70 % (NH_4) SO_4 precipitate (after dialysis)	32	23	51	13	222000	0 565	22	26	80	96
V Gel filtration on Sephadex G 100 (fractions 21 28)†	42	12	32	8	268000	0 670	14	15	96	112

* Diffusion units determined by the CP method

§ Units determined by the method of *Kunitz*

† Fractions 21–28 of ten runs were poured together dialysed against distilled water and concentrated by freeze drying to 42 ml

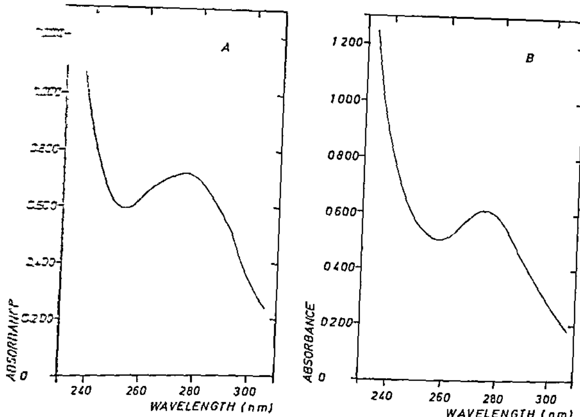


Fig 4 Ultraviolet absorbtion spectrum of the purified proteinases A and B in 0 05 M acetate buffer pH 5 6

obtained with all the inhibitors examined While no differences were observed regarding the inhibitors in blood sera, the inhibitors from wheat, corn and rye inhibited proteinase A, but not proteinase B The other inhibitors of animal and vegetable origin reacted in the same way with both of the proteinases

Molecular weights The molecular weights of the proteinases were determined by the Sephadex gel filtration method (*Whitaker* 1963), with Blue dextran, bovine serum albumin, ovalbumin, bovine α chymotrypsin, pepsin and cytochrome C as reference substances The enzymes were eluted from the columns as single peaks, and proteinase A behaved as if it had a molecular weight of 22 100 while proteinase B behaved as if it had a molecular weight of 43 600

DISCUSSION

The amounts of extracellular proteinases produced by *Ae liquefaciens* and *Ae salmonicida*, when grown for 60 to 80 hours at 30° C on semi solid *skim milk agar* were of considerable size, as shown in Fig 1 and Table 1 The table also shows that the concentrations of protein in the proteinase solutions were relatively low and consequently, the specific activities were high Thus, the extracellular aqueous phase containing metabolical components and break down products of the media was well suited as starting material for the purification of the two enzymes

As seen from Table 2 and Table 3, the described five step purification procedure results in approximately a hundred fold purification of the two enzymes The purity and homogenicity of the preparations are attested by the electrophoretic and chromatographic

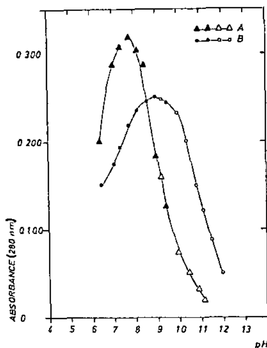


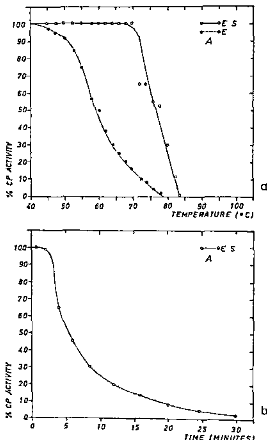
Fig 5 Optimum pH of the proteinases A and B 0 05 ml enzyme solution was added to a mixture of 1 ml 2 per cent casein solution and 3 ml buffer, and incubated for 20 minutes at 37° C The reaction was terminated by the addition of 3 ml trichloracetic acid and the absorbance at 280 nm in the supernatants was used as a measure of the activity ▲——▲ and ●——● Tris HCl buffer △——△ and ○——○ glycine NaOH buffer

data, as well as by the high specific activities The recovery is also considered to be of an acceptable size as compared with purification procedures for proteinases of related organisms (*Wilkes et al* 1969)

Although the pH has a marked influence on the rate of enzymatic reactions, microbial proteinases are described as being active over wide pH ranges (*Hiramatsu* 1967 *Juffs* & *Doelle* 1968) This was also the case with the present proteinases, as they were found to be active against casein, at least from pH 5 to pH 12, with optima at pH 7 9 and 9 0 for A and B respectively The curves in Fig 5 are drawn continuously from the region of Tris buffer to that of glycin buffer, as the change of buffer did not seem to influence the activities This could not have been done, however, if the buffers had exerted

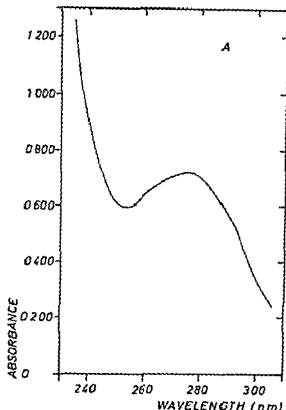
inhibiting effects upon the enzymes (*Stormer* 1968), as was the case with phosphate buffer The results could then give false optimum or minimum values

Both proteinases were found to be more resistant to heat in the presence, than in the absence of casein substrate (Figs 6 and 7) This corresponds to the findings of other authors (*Sandvik* 1962), and was not surprising The thermal inactivation of the en



Figs 6 a and b The influence of heat on the CP activity of proteinase A as (a) function of temperature and (b) function of time at 70° C Purified enzyme solutions (E) and purified enzyme solutions mixed with an equal volume of 2 per cent sodium caseinate solution (ES) were transferred in amounts of 0 1 ml to thin walled 1 ml glass ampules and heated as described in METHODS Aliquots of 0 025 ml were transferred to wells in sodium caseinate agar plates and the CP activity was determined on the basis of the zone diameters

nes



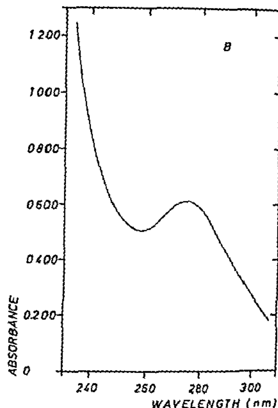


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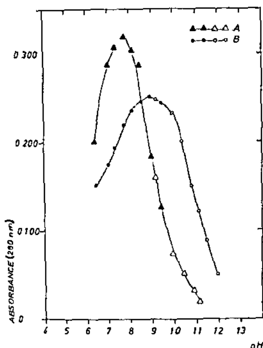
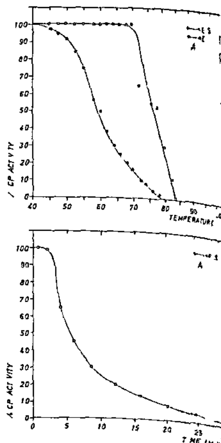


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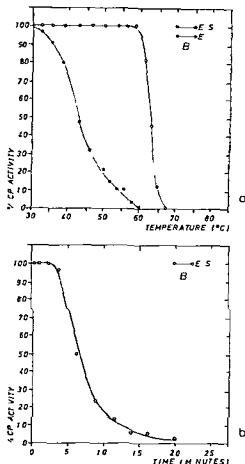
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Figs 7 a and b The influence of heat on the CP activity of proteinase B as (a) function of temperature and (b) function of time at 60° C For further details see Fig 6

zymes, in the presence of substrate is of particular interest for food science, because of the importance of heat treatment in food preservation It should be emphasized that one of the two enzymes (A) was not inactivated by temperatures commonly used for pasturization (*Jebsen* 1960), although one may expect that the corresponding organisms are killed at lower temperatures

As the inhibition and activation of reaction rates by EDTA and divalent metal ions are common features in reactions catalysed by bacterial proteinases (*Wilkes et al* 1969 *Tsuru et al* 1964, *Jónsson* 1969) the present results are somewhat unusual in that the activation by cobalt ions could be controlled by the addition of EDTA while the effect of

TABLE 4 *Effects of EDTA and Some Divalent Metal Ions on the Activities of the Proteinases A and B*

Added*	Ratio of activity to original activity in the solutions of	
	proteinase A	proteinase B
$CaCl_2 \cdot 2H_2O$	1	1
$CoCl_2 \cdot 6H_2O$	28	22
$CuSO_4 \cdot 5H_2O$	1	1
$FeSO_4 \cdot 7H_2O$	26	34
$MgSO_4 \cdot 7H_2O$	1	1
$MnSO_4 \cdot 4H_2O$	1	1
$NiNO_3 \cdot 6H_2O$	1	1
$ZnSO_4 \cdot 2H_2O$	1	1
$CoCl_2 \cdot 6H_2O$, EDTA	0 1	0 3
$FeSO_4 \cdot 7H_2O$, EDTA	60	70
EDTA	0 5	1

* The metal ions and EDTA were added to a concentration of $1 \cdot 10^{-3}$ M in the enzyme buffer mixtures after which an equal volume of a 2 per cent substrate solution (casein) was added The activities were then determined by the method of Kunitz The results are expressed as multiples of the activities in the original solutions

TABLE 5 *Inhibiting Effects of Some Naturally Occurring Proteinase Inhibitors on the Activities of the Proteinases A and B*

Inhibitors isolated from	Inhibition* proteinase A	proteinase B
Horse serum	+ + +§	+ + +
Chicken serum	+ +	+ +
Rabbit serum	+ +	+ +
Cattle serum	+ +	+ +
Swine serum	+ +	+ +
Barley	—†	—
Wheat	+	—
Corn	+	—
Rye	+	—
Potato	—	—
Soy bean	—	—
Lima bean	—	—
Chicken egg white	—	—
Pancreas	—	—
Ascaris suum	+ + +	+ + +

* The results were obtained by the crosswise CPI test of *Fossum* (Fig 8)
§ + + + and + + + designate increasing degree of inhibitory effect
† — designates no inhibitory effect

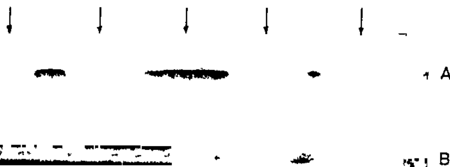


Fig 8 Demonstration of proteinase inhibitors by the crosswise CPI test Filter paper strips (3 mm wide) moistened with solutions of inhibitors were placed on sodium caseinate agar plates which were incubated for 3 hours at 37° C After removal of these strips similar strips moistened with the proteinases A and B were applied to the surface of the agar gel at right angles to the direction of application of inhibitors and the plates were incubated for a further 6–18 hours at 37° C Proteolytic activity is indicated by the white zones on the clear agar surface and the inhibition is indicated by interruption of the white precipitation zones Inhibitors are (from left to right as marked by arrows) wheat corn barley horse serum and cattle serum

iron ions was further increased by the same reagent (Table 4) This makes the use of the term metalloenzyme unsuitable until more detailed examination may be carried out on the enzymes in question

The physiological significance of the naturally occurring proteinase inhibitors is still a philosophical problem However, when characterizing and comparing the two *Aeromonas* proteinases, the crosswise CPI test of Fossum (1970), carried out with various inhibitors is considered to be of importance Table 5 and Fig 8 show some differences with regard to the effect of various inhibitors against the two proteinases, and these findings may indicate differences in the natures of the enzymes

Molecular weights for severals microbial proteinases are now known and the order of the presented values for proteinase A and proteinase B of the aeromonads corresponds to those already described (*Tsuru et al* 1964 *Jonsson* 1969 *Griffin* & *Prescott* 1969) The proteinases A and B both of which can be produced by one and the same organism (*Ae liquefaciens*) under certain environmental conditions were shown previously to be distinguishable by a serological procedure (*Dahle* 1969b), an electrophoretic zymogram technique and a combination of these methods (*Dahle* 1970) The proteinase A was also shown to possess a serological relationship with a proteinase of *Vibrio comma* (*Sandvik* & *Dahle* 1971, *Dahle* & *Sandvik* 1971), while no relationship with proteinases of other organisms has yet been reported for the proteinase B The possibility that the two enzymes constitute a monomer and a dimer of the same substance, based upon the molecular weights only, seems, therefore unlikely

The present results are insufficient to permit an understanding of the question, why does an organism sometimes produce more than one proteinase? However, the results will in combination with the fact that the environmental conditions are decisive for the production of the enzymes (*Dahle* 1969b) be of value for further studies on the function of these enzymes by the organisms in question It remains then to clarify whether the proteinases are of an induced type or not

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duced independently of inducing components, and induced enzymes which are only produced in the presence of an inducer (*Rose* 1968) In connection with the proteinase production in a *Micrococcus McDonald* & *Chambers* (1966) used the term "partially constitutive' when the proteinase formation was controlled by a form of end product induction

The close relationship between the proteinases produced by *Ae liquefaciens* and *Ae salmonicida* makes their formation of particular interest The aim of the present work was to study the regulation of the biosynthesis of the proteinases produced by the two aeromonads on the addition of certain nutrients to a basic medium

MATERIALS AND METHODS

Strains

The strains used were *Aeromonas liquefaciens* ATCC 14715, and *Aeromonas salmonicida* ATCC 14174, obtained from the American Type Culture Collection, Rockville, Maryland, U S A

Medium

The following ingredients in distilled water constituted the basic medium NH_4Cl 5 g, NH_4NO_3 1 g Na_2SO_4 2 g K_2HPO_4 3 g KH_2PO_4 1 g $MgSO_4$ $7H_2O$ 0 2 g H_3BO_4 6 10^{5} g MoO_3 3 10^{5} g, $Fe(NH_4)_2(SO_4)_2$ 0 6 10^{3} g, $MnSO_4$ $4H_2O$ 0 8 10^{3} g $CuSO_4$ $5H_2O$, 4 10^{3} g and $ZnSO_4$ $7H_2O$ 88 10^{3} g The volume was adjusted to 1000 ml The pH of *this medium was 6 2*

The following ingredients were added to the basic medium Peptone (Difco*, Bacto peptone 0118 01) neopeptone (Difco 0119 01) casein of Hammarsten quality (Merck†) sodium caseinate (Eastman Kodak§) trypsinised casein (Merck) and gelatin (Gelita°) as indicated in Table 2 The amino acids listed in Table 1 were all obtained from Nutritional Biochemicals Corporation Cleveland Ohio U S A while glucose and glycerol were obtained from Merck

The added substances were weighed directly into tubes containing 5 ml of the basic medium and the complete medium sterilized at 121° C for 15 minutes

The peptone water used consisted of 1 0 per cent peptone and 0 5 per cent NaCl in distilled water and the gelatin medium of 1 0 per cent peptone 0 5 per cent meat extract 0 5 per cent NaCl and 15 per cent gelatin in distilled water

Experimental conditions

The organisms were inoculated by adding 50 µl of 18 hours cultures into 5 ml volumes of medium in 20 ml glass tubes and the cultures incubated at 30 C Samples of the cultures were taken after 18 and 42 hours for measurement of the absorbance at 590 nm in a Hilger* spectrophotometer, and for proteinase determination

Proteinase assay

The concentrations of the proteinase were determined by the Casein Precipitation method (CP method) of *Sandvik* (1962), as modified by *Dahle* (1969a) A hot solution containing 1 40 per cent agar (Bacto agar 0140 01 from Difco) 1 00 per cent sodium caseinate (Eastman Kodak added as 4 per cent solution of pH 6 2), 0 01 per cent merthiolate and 0 004M $MgCl_2$ in distilled water, was poured into a glass tray to a depth of 2 mm and allowed to solidify Aliquotes of 25 µl from serial 2 fold dilutions of the proteinase solutions, were transferred to wells of 7 mm diameter in the sodium caseinate-containing agar before incubation at 37° C for 16 hours The estimation of diffusion units was based on the diameters of the precipitation zones which developed (*Dahle* 1969a)

Identification of the proteinases

The proteinases produced by *Ae liquefaciens* were identified by the enzymoserological Casein Precipitation Inhibition test (CPI test) (*Dahle* 1969b) *as proteinase A and proteinase B* while the one produced by *Ae salmonicida* was identified as proteinase B The CPI test is based on the separation of the antiproteinases in immune sera from normal proteinase inhibitors by paper electrophoresis Ioll wed by demonstration ǒt mi lu ıon of the Casein Precipitation reaction in the area of the antiproteinases

Zymograms of the proteinases in agar gel were produced as described by *Dahle* (1970)

RESULTS

In the basic medium used only *Ae liquefaciens* was able to grow slightly, but without the production of any proteinases After the

* Difco Laboratories Inc., Michigan, U S A
† Merck, Darmstadt, Germany
§ Eastman Kodak, Rochester, N Y , U.S.A
° Gelita Göppingen, Germany

* Hilger & Watts Rank Precision Industries Ltd London England

TABLE 1 *The Effect on Growth and Proteinase Production of* Aeromonas liquefaciens *and* Aeromonas salmonicida *by Various Ingredients Added to the Basic Medium after Adjustment of the pH to 7 5*

Medium	*Ae liquefaciens* (18 hours)		*Ae salmonicida* (42 hours)	
	Growth*	Proteinases†	Growth	Proteinases
Basic medium (Bm)	0 025	—	0 000	–
Bm + DL alanine (0 5 per cent)	0 030	—	0 000	–
Bm + L arginine (0 5 per cent)	0 035	+	0 000	–
Bm + DL-asparagine (0 5 per cent)	0 090	+	0 010	–
Bm + glycin (0 5 per cent)	0 025	—	0 000	–
Bm + L glutamic acid (0 5 per cent)	0 050	—	0 000	–
Bm + L histidine (0 5 per cent)	0 110	+	0 020	–
Bm + DL-methionine (0 5 per cent)	0 030	—	0 000	–
Bm + DL phenylalanine (0 5 per cent)	0 035	—	0 000	–
Bm + DL tryptophan (0 5 per cent)	0 035	—	0 000	–
Bm + monosodium glutamate	0 350	+ +	0 120	–
Bm + glucose (0 5 per cent)	0 140	(+)	0 000	–
Bm + glycerol (0 5 per cent)	0 080	—	0 000	–
Bm + peptone (0 5 per cent)	0 460	+ + + +	0 180	+ +

* The growth is given as the absorbance of the cultures at 590nm as compared with the absorbance of the basic medium

† The amounts of proteinases produced were determined by the CP method

+, + + and + + + designate increasing amounts of the proteinases

– designates no CP reaction

addition of certain simple compounds to the basic medium the rate of growth increased greatly, although there was still no proteinase production By adjusting the pH of the medium to above 7 0 with Tris HCl buffer the production of proteinases could be induced (Table 1) However, *Ae salmonicida* was not able to produce proteinases until more complex substances, such as peptones were added to the basic medium although slight growth was observed after the addition of some simple components (Table 1)

The table also show that monosodium glutamate was the most efficient substance among the simple compounds, for the induction of growth of both the organisms Among the simple compounds, the glutamate was also the most potent inducer of proteinase production from *Ae liquefaciens,* although the effect was much weaker than that of peptone Fig 1 shows the growth and proteinase production as functions of the glutamate concentrations for *Ae liquefaciens* The optimum concentration for proteinase production is seen to be lower (2 per cent) than the optimum concentration for growth (3 per cent)

In order to examine for differences in proteinase induction caused by proteins of higher molecular weight (casein and peptone), and lower molecular weight compounds obtained from these, or other proteins (trypsinised casein, neopeptone and gelatin) (*Difco manual* 1953, *Hildrum & Næss* 1971), these compounds were added to the basic medium to a final concentration of 1 0 per cent, and the proteinases produced were identified by the CPI-test and the zymogram technique Table 2 shows that the lower molecular weight polypeptides were the most potent inducers of proteinase biosynthesis for both the organisms, while the addition of casein (Hammarsten quality or sodium caseinate) to the basic medium did not result in proteinase production at all, although growth was moderately increased Peptone induced

duced independently of inducing components, and induced enzymes which are only produced in the presence of an inducer (*Rose* 1968) In connection with the proteinase production in a *Micrococcus McDonald* & *Chambers* (1966) used the term partially constitutive" when the proteinase formation was controlled by a form of end product induction

The close relationship between the proteinases produced by *Ae liquefaciens* and *Ae salmonicida* makes their formation of particular interest The aim of the present work was to study the regulation of the biosynthesis of the proteinases produced by the two aeromonads on the addition of certain nutrients to a basic medium

MATERIALS AND METHODS

Strains

The strains used were *Aeromonas liquefaciens* ATCC 14715 and *Aeromonas salmonicida* ATCC 14174 obtained from the American Type Culture Collection Rockville Maryland U S A

Medium

The following ingredients in distilled water constituted the basic medium NH_4Cl 5 g NH_4NO_3 1 g Na_2SO_4 2 g K_2HPO_4 3 g KH_2PO_4 1 g $MgSO_4 \cdot 7H_2O$ 0 2 g H_3BO_3 6 10^{5} g MoO_3 3 10^{5} g $Fe(NH_4)_2(SO_4)_2$ 0 6 10^{3} g $MnSO_4 \cdot 4H_2O$ 0 8 10^{3} g $CuSO_4 \cdot 5H_2O$ 4 10^{8} g and $ZnSO_4 \cdot 7H_2O$ 88 10^{4} g The volume was adjusted to 1000 ml The pH of this medium was 6 2

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The added substances were weighed directly into tubes containing 5 ml of the basic medium and the complete medium sterilized at 121° C for 15 minutes

The peptone water used consisted of 1 0 per cent peptone and 0 5 per cent NaCl in distilled water and the gelatin medium of 1 0 per cent peptone 0 5 per cent meat extract 0 5 per cent NaCl and 15 per cent gelatin in distilled water

Experimental conditions

The organisms were inoculated by adding 50 µl of 18 hours cultures into 5 ml volumes of medium in 20 ml glass tubes and the cultures incubated at 30 C Samples of the cultures were taken after 18 and 42 hours for measurement of the absorbance at 590 nm in a Hilger* spectrophotometer and for proteinase determination

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The proteinases produced by *Ae liquefaciens* were identified by the enzymoserological Casein Precipitation Inhibition test (CPI test) (*Dahle* 1969b) as proteinase A and proteinase B while the one produced by *Ae salmonicida* was identified as proteinase B The CPI test is based on the separation of the antiproteinases in immune sera from normal proteinase inhibitors by paper electrophoresis followed by demonstration of inhibition of the Casein Precipitation reaction in the area of the antiproteinases

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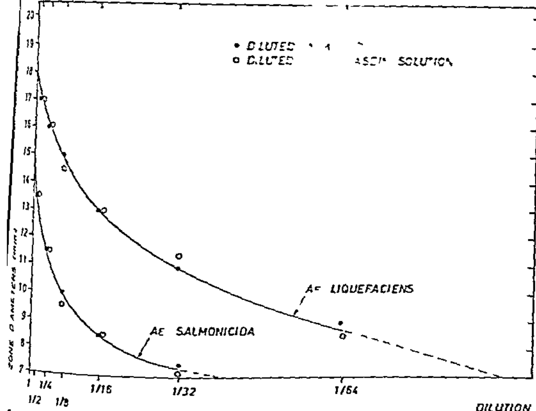


Fig 3 Zone diameters as functions of dilutions for culture filtrates of *Ae liquefaciens* and *Ae salmonicida* in water and 2 per cent casein solution (CP method The dotted lines indicate extrapolation to the endpoint dilutions

Furthermore it was not possible to induce the biosynthesis of proteinase A in gelatin medium on the addition of aminoacids This places gelatin in a very special situation regarding bacterial proteinase biosynthesis and it should be considered in connection with the extensive use of the gelatin hydrolysing test in bacterial taxonomy (*Breed et al* 1957)

In addition to the composition of the medium the environmental conditions are of importance for proteinase biosynthesis This was clearly demonstrated by the pH-effect in the basic medium used in the present study but temperature and atmospheric conditions may also be critical for the production of the proteinases (*Juffs* et al 1968)

The demonstrated differences in proteinase production in various media should not be interpreted as reducing the taxonomical value of proteinase production as suggested by *Liu & Hsieh* (1969), but rather as a recommendation to carry out the examinations for proteinase production in defined media and under standard conditions regarding pH incubation temperature and incubation time On the other hand knowledge of the requirements for microbial proteinase production is of value for food technologists in determining ideal preservation conditions for food

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Acta path microbiol Scand Section B 79, 747–753, 1971

INACTIVATORS OF HAPTEN-COUPLED BACTERIOPHAGES IN NORMAL SERA

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Hapten conjugated bacteriophages were inactivated by normal human rabbit pig and rat sera, but not by germ free piglet sera There was great individual variation in the inactivation titres As a rule the inactivators of NIP phage had higher titres than those of NP DNP, oxazolone or penicillin coupled phage This was probably due to the greater sensitivity of the NIP phage

Hapten coupled bacteriophage inactivation is a sensitive method for determining anti hapten antibodies It has been used successfully to detect antibodies whose concentration is low, such as in the primary response *in vitro* (*Segal* 1970) and in the production of antibody by single cells (*Makela* 1967)

Inactivators of hapten coupled bacteriophages have been reported in normal sera of some mammalian species (*Mäkelä* 1966 *Haimovich et al* 1970) and in lower vertebrates (*Leslie* & *Clem* 1969) These are probably specific anti hapten antibodies, although normal sera also contain inactivators of uncoupled bacteriophages, as shown by *Çetin* 1960 Brandriss has shown antibodies to dinitrophenol (DNP) in normal human sera using passive haemagglutination

The purpose of the present work was to detect and quantitate inactivators of various different hapten coupled phages in a number of mammalian species

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MATERIALS AND METHODS

Culture Media

Nutrient broth (Lab-Lemco, Oxoid), to which 5 0 g of NaCl and 246 mg of $MgSO_4$ per litre were added, was used as a diluent and liquid culture medium for bacteria Soft agar contained 6 g of agar per litre of medium, and bottom agar for the plates contained 20 g of agar per litre

The medium for growing phage contained the following ingredients A salt solution was first prepared which contained 80 g of Na_2HPO_4, 20 g of KH_2PO_4, 5 g of NaCl and 10 g of NH_4Cl per litre of water After autoclaving the pH was adjusted to 7 0 To each 100 ml of this solution 10 ml of 0 1 M $MgSO_4$, 10 ml of 0 01 M $CaCl_2$, 20 ml of 20 per cent glucose 20 ml of 20 per cent Bacto Casamino acids, 2 ml of 1 per cent trypto phan and 840 ml of water were added

Preparation of Phage Stock

To prepare the phage stocks, the phage medium was poured into Roux bottles, 250 ml into each *E coli* B was grown overnight in the phage medium and 0 25 ml of this culture was inoculated into each bottle After shaking in a nearly horizontal position at 37° C for 4 hrs the bacterial count was 5 × 10⁷/ml

One drop of phage suspension containing 100 plaque forming units (PFU) was grown for 3 hrs

on an agar plate at 37° C This culture of one drop was put into each Roux bottle and shaken for an additional 3½ hrs at 37° C by which time the suspension had become clear Then 1 ml of chloroform was put into each bottle After 15 min more shaking at 37° C the phage in the bottles was titrated The concentration was c 10^{11} PFU per ml in all bottles After filtering through filter paper the phage preparation was centrifuged twice at 5000 rev/min for 30 min, filtered through sintered glass and concentrated with a Diaflo filter PM 30 For preservation chloroform was added

Haptens

NIP (4 hydroxy 3 iodo 5 nitrophenylacetic acid) NP (hydroxy 3 nitrophenylacetic acid) and NNP *(4 hydroxy 3 5 dinitrophenylacetic acid) were prepared as described by Brownstone et al* 1966 2 4 Dinitrobenzenesulphonic acid sodium salt was *obtained from* Eastman Organic Chemicals Co *Oxazolone (2 phenyl 4-ethoxy methylene oxazolone)* was prepared by *Kari Juntunen* in this Department *Penicillin G was obtained from* Farbwerke Hoechst Ag (Frankfurt)

Preparation of Hapten Phage Conjugates

For NIP phage conjugate bacteriophage T was diluted in 3 per cent Na bicarbonate to a final concentration of 2 6 × 10^{11} plaque forming units (PFU) *per ml To 5 ml of this suspension was* added 2 2 mg of NIP azide dissolved in 0 11 ml of dimethylformamide The reaction mixture was *rocked at room temperature for 8 hrs and dialyzed* against 0 9 per cent saline containing 0 01 per cent EDTA Finally the phage suspension was *filtered through sintered glass (G-5), and a 1/10* volume of a solution containing 1000 IU per ml of penicillin 10 mg per ml of streptomycin and *10 mg per ml of BSA (bovine serum albumin)* was added 94 per cent of the phages were killed during the coupling procedure

NP *was coupled to bacteriophage* T_4 *by the* same method except that 7 mg of NP azide to 5 ml of phage suspension was used The inactiva *tion was 90 per cent*

Oxazolone was coupled o phage as described earlier by *Jormalainen et al* 1971 Bacteriophage T_4 *(3 4 × 10^{10} PFU/ml) in 0 1 M phosphate buffer pH 8 5 was reacted with 0 15 mg/ml of* oxazolone for 20 hrs at +4° C with continuous *stirring The unreacted oxazolone crystals were* spun off at 2000 rev/min for 20 min Phage suspensions were dialyzed against 0 9 per cent saline *containing 0 02 per cent Na azide and 0 01 per* cent EDTA and filtered through sintered glass Inactivation was 90 per cent

TABLE 1 *HPI Titres in Normal Human Sera*

Serum	NIP*	NP*	DNP	OX	Pen
LI	720	100	39	11	<10
AK	110	37	8§	<10	<10
RR	1000	120	5§	<10	<10
CGG	1900	110	42	<10	<10
TR	540	2§	19	<10	<10
KJ	610	27	6§	<10	<10
AS	330	93	50	<10	<10
LV	2800	33	50	<10	<10
PA	800	120	84	<10	18
HK	2000	120	46	77	<10
GM	1000	770	5§	<10	10
SAK	1700	180	48	16	< 3
KS	1800	280	26	< 3	< 3
MH	1900	64	7	15	< 3
PP	1200	56	29	< 3	< 3
AA	1700	660	10	15	< 3
OVR	1100	15	4	< 3	< 3
VV	560	69	9	< 3	< 3
HS	2600	48	28	< 3	< 3
ER	330	28	11	< 3	< 3
Geom mean	960 ×/÷ 2 25	72 ×/÷ 3 72	18 ×/÷ 2 62	9 5 ×/÷ 2 01	not calculated

* Related compounds

§ Values obtained by extrapolating

TABLE 2 *HPI Titres in Normal Rabbit Sera*

Serum	NIP	NP	DNP	OX
1	330	170	69	[illegible]
2	180	180	40	2§
3	690	180	25	[illegible]§
4	670	69	8§	1[illegible]
5	1400	120	17	[illegible]3
6	980	110	25	22
7	1500	320	39	2[illegible]
8	2500	150	10	1[illegible]
9	830	170	37	2[illegible]
10	950	88	10	3[illegible]
11	950	85	44	5[illegible]
12	530	150	8§	27
13	1700	180	14	12
14	1000	330	10	3[illegible]
15	1700	180	10	35
16	440	300	16	17
17	500	370	9§	11
18	4000	280	13	29
19	830	140	16	78
20	310	20	5§	1§
21	1200	180	10	16
22	370	180	42	81
23	360	150	42	20
24	530	120	48	3[illegible]
25	910	240	15	91
26	840	130	33	53
27	400	240	23	6§
28	650	180	17	22
29	670	240	74	39
30	850	960	12	200
31	940	280	3§	8§
32	460	35	2§	9§
33	260	53	4§	8§
34	560	89	20	26
35	400	150	8§	6§
36	830	240	17	1§
37	720	500	8§	9§
38	540	770	9§	72
39	1100	140	3§	8§
40	1800	130	6§	59
41	460	67	18	22
42	1300	87	9§	46
Geom mean	750 ×/÷ 1 83	160 ×/÷ 2 07	14 ×/÷ 2 30	22 ×/÷ 4 07

§ Values obtained by extrapolating

For DNP T_4 conjugation bacteriophage T_4 was diluted to a final volume of 3 7 × 10^{10} PFU per ml in 2 per cent carbonate buffer 2 5 ml of 8 per cent d nitrobenzenesulphonic acid dissolved in 4 per cent Na_2CO_3 (pH 0 65) was added to 1 5 ml of phage suspension The reaction mixture was shaken at room temperature for 8 hrs before dia lysis The phage preparation was not filtered cause the number of viable phages was four drop during filtration For preservation Na[illegible] 0 02 per cent was added The inactivation 94 per cent

Penicillin was coupled to bacteriophage according to the method described by *Haimo*

et al 1967 Bacteriophage was diluted to 3.4×10^{10} PFU per ml in 0.1 M carbonate buffer pH 9.5, which contained 100 mg/ml of penicillin G. The reaction mixture was shaken at 37° C for 24 hrs. Then an equal volume of 0.9 per cent saline containing 0.02 mg/ml of gelatin was added and the phage suspension was dialyzed against 0.05 M phosphate buffer pH 6.8 which contained 0.01 per cent EDTA. The inactivation was 66 per cent.

Sera

Normal sera were taken from 20 healthy persons, 20–40 years of age, who had no known contacts with the haptens in question except penicillin, from 42 normal rabbits, 36 rats, 4 guinea pigs and 2 pigs. Germ free piglet sera were kindly provided by Dr *Helena Tlaskalová* from the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague. Newborn colostrum free piglet sera were taken immediately after birth before the piglets had begun to suckle.

For the immune sera rabbits were immunized intraperitoneally with 2 mg of alum precipitated NIP-conjugated chicken globulin (NIPcg) or NPcg with 10^{10} Pertussis bacteria. Sera were taken 14 days after the injection.

Preparation of Hapten Protein Conjugates

For the Farr assay radioactive NIP BSA and NP BSA were prepared. BSA was trace labelled as described by *Sonoda & Schlamowitz* 1970. To 30 ml of 0.2 per cent BSA in 0.02 M phosphate buffer pH 7 was added 2 mCi $Na^{125}I$ and 15 ml of 133 μM chloramin T at 4° C with strong stirring. After incubation for 30 min at 4° C with stirring 3.6 ml of 667 μM $NaHSO_3$ was added and after a further 5 min 0.6 ml of 1.0 M KI was added. The solution was passed through a column containing 6 ml of AG 1 X8 anion exchange resin equilibrated with the phosphate buffer. After washing with 5 ml of the phosphate buffer more than 50 per cent of the radioactivity was in the effluent. 0.6 ml of 0.1 M KI was added. The solution was concentrated by pressure dialysis to 6 ml.

Haptens NIP and NP were coupled to radioactive and to cold BSA by the same method as described for hapten phage couplings. The molar ratio of hapten (NIP or NP) to protein was measured by optical density at 430 mμ (*Brownstone et al* 1966).

Determination of Haptenated Phage Inactivation (HPI) Titres

A 3.16 fold dilution series was made in broth from the serum. 0.3 ml of each dilution and 0.3 ml of phage suspension containing c. 300 PFU were incubated for 5 hrs at 37° C in a water bath. 2 ml of soft agar and c. 10^8 *E. coli* bacteria were added to each tube. The contents were poured onto solid 2 per cent agar in a Petri dish. The plaques formed were counted after incubation at 37° C overnight. The control tubes contained 0.3 ml of broth instead of serum.

The HPI titre was determined using the following formula

$$y = y_1 + \left[(y_2 - y_1) \Big/ (z_2 - z_1)\right] (1 - z_1)$$

where

$$z_i = \frac{x_i}{100 - x_i} \qquad i = 1, 2$$

x_i = per cent of inactivated phage in serum dilution y_i

x_n = per cent of inactivated phage in serum dilution y

titre = $1/y$

Only dilutions inactivating 10–90 per cent of the phage were used for the calculation of titres.

RESULTS

HPI Titres in Normal Sera

The individual titres of human sera are shown in Table 1. The geometric mean titres were calculated because the distribution was close to normal when the figures were plotted on a logarithmic scale. The highest titres were in the group of NIP phage inactivators, the geometric mean was 960. The mean titres of NP, DNP- and oxazolone phage inactivators were 72, 18 and <8.7 respectively. The mean titre of penicillin phage inactivators was not calculated, because only one serum gave a measurable value. The correlation coefficients gave no indication that titres to different haptens tended to be all high or all low in the same individual.

In normal rabbit sera (in detail in Table 2) the geometric mean titres were of the same order of magnitude as in human sera. However the inactivators of NP phage had higher titres than in human sera, the mean titre was 160. It differed significantly from the mean titre in human sera with a P value of 0.025. In rabbit sera also there was no correlation between different titres in the same rabbit.

TABLE 3 *HPI Titres in Normal Rat Sera*

Serum	NIP	NP	DNP	OX
1	1900	9 5	18	92
2	770	10	74	<3
3	840	8 3	19	11
4	1800	27	<3	<3
5	1400	7 4	6 7	<3
6	1200	3 2	7 4	<3
7	10000	48	4 5	<3
8	5000	8 7	28	24
9	690	11	12	<3
10	3300	36	<3	<3
11	1800	5 1	42	<3
12	770	9 5	<3	<3
13	630	33	16	<3
14	1300	91	3 3	<3
15	1300	50	<3	<3
16	480	5 0	10	<3
17	910	5 3	<3	31
18	650	5 6	36	<3
19	800	12	10	<3
20	1300	25	150	<3
21	1100	22	<3	<3
22	500	43	28	<3
23	1200	540	140	13
24	830	28	6 3	<3
25	1700	29	22	<3
26	710	8 7	4 6	<3
27	500	10	<3	<3
28	380	3 3	6 7	<3
29	670	20	29	<3
30	360	29	<3	4
31	240	59	34	<3
32	330	13	18	<
33	53	<3	<3	<3
34	10	<3	<3	<
35	170	14	<3	<3
36	640	<3	<3	<
				<3
Geom mean	714 x/— 3 26	<13 x/— 3 73	<8 4 x 3 9	[illegible]

The titres of NP-phage inactivators in rat sera differed clearly from those in rabbit sera the P value was below 0 001 (Table 3) The mean titre of oxazolone phage inactivators was lower than in rabbit sera the P value was below 0 001 In rat sera the correlation between the titres of NIP and NP phage inactivators and NP and DNP phage inactivators was significant

Five human and five rabbit sera were tested with unconjugated phage All titres were below 3 as [illegible] method in question This result [illegible] to the result obtained with [illegible] in human sera where [illegible] below the lower limit of the [illegible] were tested with [illegible] pig sera and T_4 In the [illegible] phages T_2 96 per cent of T_2 [illegible] (1 2) 41– phages were [illegible] per cent of T_4 the results [illegible] is similar to

Nine germ fre[illegible] normal sera [illegible] were tested for

IMMUNE PRECIPITATION OF NOT CELL-BOUND ANTIGEN OF *MYCOPLASMA PNEUMONIAE*

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A precipitin reaction with not cell bound antigen of *Mycoplasma pneumoniae* is described Immune precipitates are formed with human and rabbit anti *M pneumoniae* sera in fluid medium, whereas attempts at precipitation in agar gel were negative The immune precipitation proceeds well without fresh serum, but the addition of 5 per cent fresh guinea pig serum to the reaction mixture increases the sensitivity of the reaction Rising precipitin titres are demonstrated in patients with pneumonia caused by *M pneumoniae*

Not cell bound complement fixing antigen (NCBA) of *M pneumoniae* is demonstrable in filtrates from broth cultures passed through filters which effectively retain viable cells (3, 4) NCBA can be measured in the filtrates by a direct complement fixation (CF) measurement technique (2, 3), and it can be precipitated from the filtrates with ammonium sulphate (4) The time pattern of NCBA in broth culture has been studied (4) In the present paper the precipitation of NCBA with specific antisera against *M pneumoniae* is reported Rising titres of precipitating antibodies have been demonstrated in patients suffering from pneumonia caused by *M pneumoniae*

MATERIAL AND METHODS

Preparation of precipitating antigens Immune precipitates of NCBA were produced from native filtrates from broth cultures of *M pneumoniae* and from NCBA preparations concentrated and purified through precipitation of native filtrates with $(NH_4)_2SO_4$ Data regarding the yield and degree of purification of NCBA obtained with the $(NH_4)_2SO_4$ precipitation technique will be published elsewhere (7) The preparation of the precipitating antigens employed in the present work was briefly as follows A broth culture of the Bård strain of *M pneumoniae* was made up in the broth medium referred to earlier (1) the inoculum employed being cells washed free from NCBA upon autoclaved Millipore ® (MF) filter discs type GS as described in (4) At day 8, i e days after NCBA was first demonstrable in the broth culture by the direct CF measurement technique, the whole broth culture was filtered through MF type GS A portion of the native filtrate was stored. The remaining volume of filtrate was precipitated with 31 per cent $(NH_4)_2SO_4$ at pH 7 to 538 ml filtrate with pH 5 7 was added 36 ml 1 M Tris maleate buffer pH 8 1 To the total volume of 574 ml having a pH of 7 0 was gradually added 101 g $(NH_4)_2SO_4$ pro analysi Merck the precipitation was allowed to proceed for 45 min at +4° C under continuous stirring The material was then centrifuged at 27 000 G for 15 min and the pellet resuspended in 18 ml of veronal buffered saline pH 7 2 (diluent) The antigen was then dialysed at +4°C against several changes of diluent until a negative BaCl test in the dialysate indicated a high degree of freedom from $(NH_4)_2SO_4$ (7) The concentrated precipitating antigen was then harvested from the dialysis tubing and stored frozen

Received 27 v 71

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(—20° C) in small portions The CF titre (inverse dilution containing one 50 per cent CF unit) of the native filtrate was 10, and that of the concentrated precipitating antigen was 140 —The concentrated precipitating antigen as used in the present study had a slight bluish white opaqueness This macroscopic appearance may be altered to water clearness by a treatment at pH 5 3 with moderate loss of antigen This purification procedure will be described in greater detail in (7) and was applied in the experiments reported in (6) — Control antigens were a filtrate from sterile PPLO broth medium, b concentrated control antigen obtained by precipitating filtrate from sterile PPLO broth medium with 31 per cent $(NH_4)_2SO_4$ and otherwise proceeding on the same lines as in preparing the concentrated precipitating antigen

Sera Employed in the Immune Precipitation Experiments

1) Hyperimmune serum and pre immunization serum from a rabbit (R1) immunized with the Bard strain of *M pneumoniae* cultivated in a broth medium made up of rabbit meat extract and normal rabbit serum and harvested by centrifugation Details are given in (1)

2) Hyperimmune serum and pre immunization serum from a rabbit (R2) immunized with the Bard strain of *M pneumoniae* in the same way as R1, except that frozen serum from the same rabbit (R2) was used as enrichment of the broth medium employed in the production of the immunizing antigen

3) Paired sera from patients with a clinical diagnosis of pneumonia and showing rising CF—titres with *M pneumoniae* cell antigen Five available serum pairs were arbitrarily chosen from our frozen stock of sera from hospitalized patients sent to this laboratory for routine diagnostic examination

4) Antisera against mycoplasmas other than *M pneumoniae*

a Hyperimmune rabbit serum against *M hominis 1* processed by Microbiological Associates Bethesda Maryland U S A

Hyperimmune rabbit sera against the following mycoplasmas were produced by the author *

b *M salivarium*

c *M orale 1 syn M pharyngis* (strain Symons)

d *M fermentans* (strain G)

e *M arthritidis* (strain Campo)

* The strains of *M salivarium M fermentans* and *M arthritidis* were kindly supplied by Dr *R H Leach*, The Wellcome Research Laboratories Beckenham, Kent England and the strain of *M orale 1 syn M pharyngis* by Dr *K Lind*, Statens Seruminstitut Copenhagen Denmark

These antisera were produced in the same way as antiserum R1 against *M pneumoniae*, except that *M salivarium*, *M orale 1 syn M pharyngis* and *M fermentans* were cultivated anaerobically in an atmosphere consisting of 95 per cent N_2 and 5 per cent CO

EXPERIMENTAL

All dilutions were made in veronal buffered saline pH 7 2 Pretreatment of sera and antigens the sera were diluted 1 10, inactivated at 56° C for 30 min and then clarified by centrifugation at 25 000 G for 90 min The native filtrate was inactivated undiluted and then centrifuged at 25,000 G for 45 min The concentrated precipitating antigen was centrifuged undiluted at 20 000 G for 45 min The precipitin reaction was set up in glass tubes 10×76 mm The reaction mixture consisted of 0 3 ml diluted antigen 0 3 ml diluent, and 0 3 ml diluted serum The components were mixed well, the tubes incubated at 37° C for 1 hour and thereafter at +4° C overnight Finally the reaction tubes were centrifuged at 17,000 G for 30 min Antigen controls (0 3 ml antigen and 0 6 ml diluent) and serum controls were included in all experiments

RESULTS

No macroscopically visible changes occurred in the test tubes as a result of the precipitin reaction with NCBA of *M pneumoniae* before the final centrifugation of the test mixture A positive precipitin reaction manifested itself in the formation of a centrifugation pellet, which, when resuspended in 0 1 ml diluent broke up in several firm aggregates of varying sizes, the greatest ones easily visible to the naked eye Negative precipitin reactions and the antigen controls and serum

Table 1a *Chessboard Titration of Not Cell bound Precipitating Antigen of* Mycoplasma penumoniae *(Native Filtrate) against Rabbit Anti* M pneumoniae *Hyperimmune Serum (R2)*

Dil of serum	Dilution of antigen 1 1	1 2	1 4	1 8	1 16	1 32
1 10	+	+	+	+	+	—
1 20	+	+	+	+	+	—
1 40	—	—	—	—	—	—

+ = formation of precipitate
— no precipitate formed

3

Fig 3 Single cell of agar grown *Mycoplasma pneumoniae* negatively stained with 0 4 per cent (w/v) sodium s licotungstate pH 6 5

suitable for EM studies The mechanism of the purification procedure may be to remove easily precipitable non mycoplasmal material which co precipitates during the formation of the immune complexes For immune EM examination of the antigen reaction mixtures without fresh serum (5) were preferred From the preceding studies (5) and the control system included in the present work the immune precipitate of NCBA of *M pneumoniae* formed with the human con valescent phase serum is considered specific in a serological sense Hence it was anti cipated that the pellet would mainly be composed of mycoplasma elements in an aggregated state It is therefore considered highly probable that the globular elements shown in Fig 1 are mycoplasmal in nature The elements have diameters ranging from 10 to 100 *nm* and they are characterized by the absence of membrane contours which are easily recognizable in the cells of the organism (Fig 3) The possibility that structures demonstrated in the EM by the negative staining technique could be arte facts (16) has been considered and found improbable in the present case The rabbit hyperimmune serum displayed a weak re activity with control antigen but the same globular elements were also found in the precipitates formed with that serum With the reservation that mycoplasma elements are prone to disconfiguration during preparative procedures it is concluded that the results of the present examination provide evidence that globular elements measuring 10–100 *nm* are produced during the growth of *M pneumoniae* in broth culture These elements do not seem to have been described in earlier ultrastructural studies of *M pneumoniae* which have been performed on the organisms grown in broth culture (9 10 11, 12 13 15) agar culture (3 14) in broth culture upon glass and plastic surfaces (2) and in HeLa cell cultures (17) The techniques employed in the present work have not been applied to morphological studies of *M pneumoniae* before The primary object of the present study has been the NCBA of *M pneumoniae* a limited examination of the cells of the organism (Fig 3) was included in the work in order to obtain additional experience with the negative staining technique in the study of *M pneumoniae* especially its suitability for the demonstration of membrane contours *Domermuth et al* (3) demonstrated rod shaped structures situated peripherally in *M pneumoniae* cells growing on solid medium, *Biberfeld et al* (2) found rod like structures of similar morphology in *M pneumoniae* organisms growing in broth on glass and plastic surfaces A bud formation has been observed in *M pneumoniae* cells by *Furness* (11) *Lipman et al* (15) and *Zucker Franklin et al* (17) In mycoplasmas other than *M pneumoniae* small globular elements have been described by several authors and play an important role in the conflicting theories on the replication of mycoplasmas discussed in the literature Their diameters are generally given as 100 nm or higher up to about 250 nm In several mycoplasmas elementary bodies represent minimal reproductive units formed during a characteristic reproduction cycle (7) Granules of similar morphology are con sidered by several authors to represent degenerative products derived from involu

tionary cells (for reviews, see 1,8) Small globular elements observed in different mycoplasmas and by different techniques may not represent the same entity The small globular elements demonstrated in the present work have been derived from *M pneumoniae* broth culture filtrates, most of which did not elicit colony formation when plated upon PPLO agar medium or acid production when inoculated into PPLO broth medium with dextrose (4) A small number of colonies developed from some of the filtrates, these are believed to stem from an occasional leakage of cells through the filtration membrane rather than from growth arising from NCBA Before definite conclusions can be drawn concerning the nature of the small globular elements, further studies of their morphology and chemical composition are needed, and it is considered that the immune precipitation technique would be a useful tool in such studies

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Fig 3 Single cell of agar grown *Mycoplasma pneumoniae*, negatively stained with 0 4 per cent (w/v) sodium silicotungstate pH 6 5

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Granules of similar morphology are considered by several authors to represent degenerative products derived from involu

ment of new antigen sensitive cells (*Sercarz* & *Byers* 1967, *Sterzl et al* 1969) and 2) a decrease in the amount of persisting primary antibodies (*Rubin* & *Spärck* 1971) Thus as time passes, a secondary response in animals challenged with a high antigen dose should be restored, whereas the secondary response in animals challenged with a low antigen dose should increase with time as memory cells with high affinity receptors mature and the affinity of serum antibodies (in decreasing amounts) increases (*Walker* & *Siskind* 1968)

In the present study groups of mice injected primarily with 4 × 10^6 or 4 × 10^5 SRBC were rechallenged with the same two antigenic doses after the lapse of 10, 30 and 90 days The results were correlated with the events during the primary immune response

MATERIALS AND METHODS

The materials and methods used in the present experiments were the same as those used in the preceding paper (*Rubin* 1971 a)

Inbred AKR mice, females 2 3 months of age were used throughout Sheep erythrocytes (SRBC) were washed three times with phosphate buffered saline (PBS) and resuspended in PBS to give 4 × 10^6 and 4 × 10^5 SRBC per 0 2 ml This volume was injected intravenously (i v) into the tail vein of the mouse Mice were bled from the retro orbital sinus using a capillary pipette

The haemolytic plaque assay was carried out according to *Wortis et al* (1966) In this report direct plaques are called 19S plaques and developed plaques 7S plaques

Haemagglutination haemolysis and gel filtration were carried out as described previously (*Rubin* 1971 a)

Antiserum pools obtained from different experimental groups of day 10 30 and 90 following immunization were tested for inhibitory activity *in vivo* All antiserum pools were titrated and tested for haemolytic and haemagglutinating antibodies They were then diluted to the same antibody concentration and 0 4 ml or 0 8 ml of antiserum dilution was injected intraperitoneally (i p) into AKR mice injected 2 days earlier with 4 × 10^6 SRBC Differences in the percentage of inhibition were taken as the relative binding activity of the sera tested (*Moller* 1969, *Rubin* unpublished data)

Geometric means of plaque forming cells (PFC) per 10^8 spleen cells of the different groups of mice were calculated according to *Wortis et al* (1966) Immunological memory was expressed as net excess secondary responses, as described by *Nossal et al* (1965)

RESULTS

Two separate experiments were performed with 108 mice divided into 18 groups of 6 mice As the results were very similar, the experiments will be presented as results of nine groups of 12 mice, designated groups I–IX Groups I, II, IV, V, VII and VIII received a primary i v injection of 4 × 10^6 SRBC, groups III, VI and IX 4 × 10^5 SRBC On day 10, groups I–III were rechallenged i v with 4 × 10^6 and 4 × 10^5 and 4 × 10^5 SRBC, respectively, as were groups IV–VI and groups VII–IX on day 30 and day 90, respectively (see also Table 1)

The primary 19S and 7S PFC response following challenge with 4 × 10^6 and 4 × 10^5 SRBC can be seen in Table 2 The figures are expressed as $\log_{10}$ PFC/10^8 spleen cells and they are geometric means of at least 20 mice Maximum 19S and 7S response in mice challenged with 4 × 10^6 SRBC was achieved on day 5, whereas maximum 19S and 7S response in mice challenged with 4 × 10^5 SRBC was achieved on day 4 and day 6, respectively

Maximum secondary 19S PFC response was obtained on day 3 and maximum 7S PFC response on day 4 following rechallenge The net excess 19S and 7S secondary responses were therefore calculated on day 3 and 4, respectively The effect of prolongation of the time interval from 10 to 90 days in mice primarily challenged with 4 × 10^6 SRBC and rechallenged with 4 × 10^6 SRBC is seen in Fig 1 The 19S memory response showed a slight increase during the first 30 days, whereafter it increased rapidly to a 19S memory on day 90, about 30 times that on day 10 The accompanying 7S memory response increased relatively constantly over the experimental period, being about 40 times higher on day 90 than on day 10 Rechallenge with 4 × 10^5 SRBC (groups II, V and VIII) showed a quite different pic-

TABLE 1 *Experimental Design*

Antigen dose Primary/secondary	Interval between primary and secondary stimulation 10 days	30 days	90 days
4 10^6/4 10^8 SRBC	Gr I	Gr IV	Gr VII
4 10^6/4 10^8 SRBC	Gr II	Gr V	Gr VIII
4 10^8/4 10^8 SRBC	Gr III	Gr VI	Gr IX

ture (Fig 1) The 19S memory response decreased rapidly from day 10 to day 30, from this time on it again increased to reach the value of day 10 on day 90 The 7S memory response was fairly constant on a very high level during the experimental period

The 19S memory response in mice primarily challenged with 4 × 10^8 SRBC and rechallenged with 4 × 10^8 SRBC (groups III, VI and IX) was negative during the experimental period, whereas the 7S memory response increased rapidly from day 10 to 30 (about 20 times) and then remained constant (or slightly decreased) The 7S haemolysin titres measured 7 days after rechallenge are in accordance with the 7S PFC results (Table 3)

In order to elucidate the role of persisting primary serum antibodies on the secondary responses just described antisera from day 10 (group I–III), day 30 (group IV–VI) and day 90 (group VII–IX) were tested for haemagglutinating and haemolytic antibodies The haemolytic titres of the nine different groups on the day of rechallenge can be seen in Table 3 Sera from groups I and II IV and V, VII and VIII were pooled and their haemolytic titres can be seen in Table 4 Pool I–II IV–V and VII–VIII and sera from groups III VI and IX were then diluted to the same haemolytic titre in BSS and 0 4 ml of antiserum dilution was injected i p into AKR mice challenged with 4 10^6 SRBC two days earlier The mean log haemolytic titre of antiserum injected was four and if the titre of an antiserum was lower more than 0 4 ml of antiserum was tested *in vivo* (see Table 4)

Injection of anti SRBC antiserum into AKR mice challenged with 4 10^6 SRBC two days earlier caused an inhibition of the 19S PFC response in these mice (measured on day 5 after antigen) Differences in the percentage of inhibition by the different anti SRBC antisera diluted to the same haemolytic titre were taken as the relative binding capacity of these antisera (*Moller* 1969 *Walker* & *Siskind* 1968 *Rubin* unpublished data) As can be seen in Table 4 the inhibitory capacity of anti SRBC antibodies from mice challenged with 4 10^6 SRBC decreased rapidly from day 10 to day 90 whereas the inhibitory capacity of anti SRBC antibodies from mice challenged with 4 10^8 SRBC increased from day 10 to day 30 and then remained constant (In order to correlate test animal groups 3–4 and 5–6 the inhibition factor of groups 5–6 should be multiplied by a factor two due to the amount of antiserum injected)

TABLE 2 *Primary Immune Response Against Two Different Doses of SRBC in AKR Mice Measured as* Log_{10}*PFC/10^8 Spleen Cells*

Antigen dose	Antibody class	Days after antigenic challenge 3	4	5	6	7
4 10^6	19S	2 624 ± 0 075	4 131 ± 0 121	4 233 ± 0 073	3 501 ± 0 109	3 271 ± 0 011
–	7S	2 060 ± 0 107	3 075 ± 0 218	3 636 ± 0 098	3 298 ± 0 149	3 156 ± 0 115
4 10^8	19S	4 565 ± 0 036	4 737 ± 0 081	4 170 ± 0 064	3 722 ± 0 069	3 663 ± 0 061
–	7S	0 000	4 653 ± 0 105	5 004 ± 0 118	5 375 ± 0 063	5 096 ± 0 068

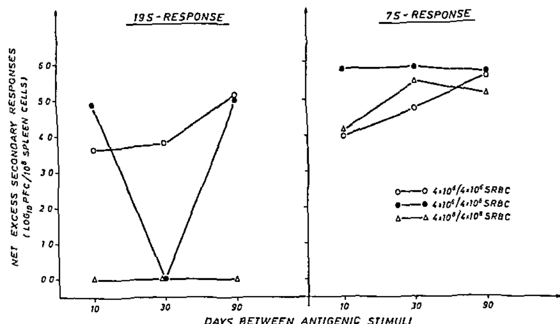


Fig 1 Summary of the cellular 19S and 7S memory responses of the different groups of mice mentioned in Results" Each point represent the geometric mean of 12 mice The 19S memory response and the 7S memory response was measured day 3 and 4 following rechallenge, respectively

DISCUSSION

Does a high saturating antigen dose prevent the animal from responding to a rechallenge due to exhaustion of memory cells or due to the inhibitory activity of endogeneously produced antibody? An increase of time after primary injection will favour 1) recruitment of new antigen sensitive cells and 2) a decrease of the amount of persisting primary antibodies The passage of time would then tend to restore the secondary antibody response (*Hege & Cole* 1966, *Sercarz & Byers* 1967, *Sterzl et al* 1969, *Rubin & Spärck* 1971)

TABLE 3 *Log₂ Haemolysin Response to SRBC in AKR Mice**

Group no	Antigen dose primary/secondary	Interval between antigenic stimuli	Days after rechallenge 0		7	
			19S	7S	19S	7S
I	$4\ 10^6/4\ 10^6$	10	4	4	9	7
II	$4\ 10^6/4\ 10^8$	–	4	3	8	7
III	$4\ 10^8/4\ 10^8$		6	6	6	6
IV	$4\ 10^6/4\ 10^6$	30	2	3	<2	8
V	$4\ 10^6/4\ 10^8$	–	2	3	<2	8
VI	$4\ 10^8/4\ 10^8$	–	<2	8	<2	10
VII	$4\ 10^6/4\ 10^6$	90	<2	3	<2	7
VIII	$4\ 10^6/4\ 10^8$		<2	2	<2	7
IX	$4\ 10^8/4\ 10^8$		<2	6	<2	10

* AntiSRBC haemolysin antibodies of 19S and 7S class were measured after gel filtration on Sephadex G200

TABLE 4 *Inhibition of the 19S PFC Response Against 4 10⁸ SRBC in AKR Mice by Passive Anti SRBC Antibody*

Test AKR mice groups	INJ with serum from AKR mice groups	Titres* at the day of SEC INJ 19S/7S	Diluted or undiluted	ML serum INJ	Log_{10} 19S PFC/10^8 spleen cells§ Normal	Serum treated	Inhibition factor
1	I II	4/4	UNDIL	0 4	4 233	3 324	8 1
2	III	6/6	1 4	0 4	–	3 726	3 2
3	IV–V	2/3	UNDIL	0 8	–	3 691	3 5
4	VI	2/8	1 16	0 4		3.356	7.5
5	VII VIII	2/2	UNDIL	0 8	–	3 988	1 8
6	IX	2/6	1 8	0 4	–	3 746	3 6

* Log_2 haemolytic titre measured following gel filtration on Sephadex G 200

§ Measured day after antigenic challenge

In the author's experiments, the main characteristics of the primary responses to 4×10^8 and 4×10^9 SRBC were as follows Maximum 19S and 7S PFC responses to 4×10^8 SRBC were demonstrated day 5 following challenge, whereas maximum 19S and 7S responses to 4×10^9 SRBC were obtained on day 4 and day 6 respectively Thus a high antigen dose delays the 7S response as compared to the low antigen dose This delay is seen also on day 3 after challenge, where the 7S PFC response to the high antigen dose was negative in contrast to the 7S PFC response to the low antigen dose (Table 2) According to the X Y Z scheme the high antigen dose leads to a rapid differentiation of antigen sensitive cells via memory cells to 19S PFC leaving few cells in the memory cell compartment to differentiate into 7S PFC (*Sterzl et al* 1969) According to *Theis & Siskind* (1968) a high antigen dose tends to make cells with receptors of high affinity tolerant Thus the day 3 7S PFC seen in mice given the low antigen dose possibly represent antibody forming cells the ancestor cells of which had high affinity receptors It is assumed that the binding characteristics of the cell receptors are reflected in humoral antibodies (*Siskind & Benacerraf* 1969 *Anderson* 1970 *Bullock & Rittenberg* 1970) Determination of the amount and avidity of persisting primary antibodies on the day of rechallenge should therefore give valuable information concerning the regulatory activity of cell bound and serum antibodies

As seen in Tables 3 and 4 the amount and inhibitory activity of antisera from mice challenged with the low antigen dose decreased from day 10 to day 90 The amount of anti SRBC antibodies in mice challenged with the high antigen dose reached a maximum on day 30 whereas the inhibitory activity of these antibodies increased from day 10 to day 30 and then remained constant (or slightly decreased) A fairly constant inhibitory activity of an antiserum during an immune response based on a summation of amount and avidity has been described previously (*Rubin* 1971b) This is not found here probably due to the low sensitivity of the *in vivo* method used An antiserum (0 4 ml) must have a log haemolysin titre of at least 4 to give reproducible results (*Rubin* unpublished data) Thus the decreasing inhibition factor of antisera from mice challenged with 4×10^9 SRBC is more a lack of sufficient antibody to test than an actual decrease in avidity

The secondary 19S and 7S cellular response to 4×10^8 SRBC in mice challenged with the same antigen dose was increasing during the experimental period This seems to correlate 1) with the cellular events during the primary immune response (selection of memory cells with receptors of high affinity for the antigen (*Anderson* 1970 *Bullock*

& *Rittenberg* 1970)), and 2) with the amount and inhibitory activity of persisting primary antibodies (both decreasing with time) The inhibitory activity of serum antibodies seems to work preferentially on 19S memory cells (see Fig 1 and Table 2) in accordance with *Uhr* & *Möller* (1968)

When mice challenged primarily with 4×10^8 SRBC were rechallenged with 4×10^5 SRBC (a dose combination favouring the 19S memory response *Nossal et al* 1965, *Wigzell* 1966, *Sercarz* & *Byers* 1967), the action of persisting primary antibodies should diminish (see 2) above), specially when the high secondary antigen dose was introduced late in the immune response A strong decrease in the 19S memory response was seen from day 10 to day 30 in accordance with the results of the workers quoted above However, very surprisingly the 19S memory response increased again and reached the day 10 value at day 90, especially when it is considered that the secondary 7S PFC response in these groups of mice remained constant at a very high level through the experimental period It has been claimed (*Nossal et al* 1965, *Wigzell* 1966, *Sercarz* & *Byers* 1967) that the 19S memory is short lived This statement is perhaps true when endogeneously produced antibodies (increasing in amount and avidity) and 19S memory cells compete for a given antigen It is suggested that whenever the inhibitory activity of serum antibodies drops below a certain level, the 19S memory can be expressed again

The regulatory role of serum antibodies is seen also in mice challenged with 4×10^8 SRBC and rechallenged with the same dose Here no 19S memory response could be demonstrated over the entire experimental period The 7S cellular and humoral memory response was restored as expected As shown in Table 4, the inhibitory activity of persisting primary antibodies increased Thus in the $4 \times 10^8/4 \times 10^5$ SRBC dose combination both cellular (the memory cell compartment differentiating into 7S PFC) and humoral factors collaborate in the depression of a 19S memory response

The recent findings of *Bullock* & *Rittenberg* (1970) extend the present suggestions on the activity on the cellular level They found 1) that cells tested for secondary 19S PFC early after priming (with TNP KLH*) were twice as responsive to a high antigen dose than cells tested late after priming and 2) that cells tested for secondary 7S PFC early after priming responded maximally to a higher antigen dose than did cells tested for 7S PFC late after priming The antigen dose yielding maximum stimulation of early 7S memory cells suppressed the response of late 7S memory cells These experimental data concerning the responsiveness of 7S PFC are in agreement with the results of *Anderson* (1970)

In a recent review *Greaves* (1970) has described experimental work which [illegible] an increasing need for cooperation of thymus derived lymphocytes in the [illegible] response to SRBC, when the antigen dose is decreased and when the time interval is increased His studies may have a bearing on the present results

* TNP Trinitrophenyl group KLH [illegible] Limpet Haemocyanin

The author wishes to thank I[illegible] reading the manuscript and [illegible] opportunity of carrying out th[illegible] discussions with friends and col[illegible] Seruminstitut are gratefully a[illegible] skilful technical assistance of M[illegible] Mr B *Aasted* is gratefully [illegible] thanks are also due to Mr F[illegible] the animals and performed the [illegible]

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CELLULAR AND HUMORAL ANTIBODY PRODUCTION AGAINST SHEEP ERYTHROCYTES IN AKR MICE

3 *Effect of Passive Antibody on the Development of Immunological Memory*

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Mice were injected with a low or a high dose of sheep red blood cells (SRBC) followed by the injection 1 or 2 days later of varying quantities of anti SRBC antiserum The effect of the passively administered antiserum on the primary and the secondary antibody response was studied It was found that a low dose of antigen was unaffected by antiserum treatment and initiated a normal primary response whereas a high antigen dose was greatly affected by antiserum treatment in the primary response Both 19S and 7S memory could be more or less inhibited by passive antiserum treatment, dependent on the quantity of antiserum injected Evidence for a 7S regulating effect on the 19S antibody synthesis is presented The results are discussed on the basis of the 'cell selection by antigen' hypothesis and a central and peripheral inhibiting effect of passive antiserum

It has been shown by various investigators that both the primary response (*Möller* & *Wigzell* 1965, *Henry* & *Jerne* 1967) and the secondary response (*Sterzl et al* 1969) to SRBC can be suppressed by injecting passively administered 7S antibody prior to simultaneously with, and even after the injection of primary antigen It has also been shown that the secondary response is much more difficult to suppress than the primary response

As shown by *Moller* (1969), passive antibody suppressed selectively the immune response induced by a high antigen dose This finding is in agreement with the "cell selection by antigen" hypothesis recently reviewed by *Siskind* & *Benacerraf* (1969), which suggested that a low antigen dose preferentially stimulates antigen sentitive cells with high affinity receptors, and that a high antigen dose stimulates cells with low affinity receptors, causing cells with receptors of high affinities to become tolerant (*Theis* & *Siskind* 1968)

The effect of 6MP and the time interval between the antigenic stimulus on the immunological memory has been described in the two previous communications in this series (Rubin 1971a, b)

The present experiments amplify the experimental design of *Möller* (1969) in order to elucidate the effect of different quantities

Received 17 v 71

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TABLE 1 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10⁶ SRBC on the Number of PFC in the Spleen Tested on Day 5 in the Primary Response*

Group	Log_2 passive antiserum titre	Days of admin	Log_{10} 19S PFC per spleen ± SE	Log_{10} 7S PFC per spleen ± SE
I	0	–	3 547 ± 0 018	–
II	9	2	3 627 ± 0 075	–
III	6	2	3 858 ± 0 096	–
IV	3	2	3 550 ± 0 097	–
V	9	1	3 833 ± 0 066	–
VI	6	1	3 719 ± 0 097	–
VII	3	1	3 443 ± 0 062	–

Six mice in each group

of passively administered antiserum (injected on day 1 and day 2 following antigenic challenge) on the immunological memory induced by a low and a high antigen dose

MATERIALS AND METHODS

Inbred AKR/Ssc male mice aged 2 3 months were used throughout Sheep erythrocytes (SRBC) were washed three times with phosphate buffered saline (PBS) and resuspended in PBS to give 4×10⁶ and 4×10⁸ SRBC per 0 2 ml This volume was injected intravenously (i v) into the tail vein of the mouse Mice were bled from the retro orbital sinus using a capillary pipette

Antibodies were produced by hyperimmunization of 100 mice as follows 2 3 weekly intraperitoneal injections of 4×10⁸ SRBC for 4 weeks then one week without any injection followed by a single injection of 4×10⁸ SRBC in the 6th week The mice were bled 7 days after this last injection

The antisera were pooled and separated into 19S and 7S components by Sephadex G 200 gel filtration Only the 7S fraction was used in these experiments Three different dilutions of antiserum were used with log_2 haemagglutination titres of 9 6 and 3 These were injected intraperitoneally in a volume of 0 2 ml

The haemolytic plaque assay was carried out according to Wortis et al (1966) The developing antiserum was prepared in rabbits by hyperimmunization with rabbit red blood cells (RRBC) coated with mouse anti RRBC (doses of complex 4 10⁸ RRBC, injected intraperitoneally) In this report direct plaques are designated '19S plaques and developed plaques 7S plaques

Haemagglutination was carried out using the microtitre equipment with 1 per cent SRBC in balanced salt solution pH 6 9 (BSS) Inactivation of antisera with 2 mercaptoethanol (2MP was carried out according to Uhr & Finkelstein 1963)

RESULTS

336 mice were divided into seven groups each containing 48 mice Half of the animals

TABLE 2 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10⁸ SBRC on the Number of PFC in the Spleen Tested on Day 5 in the Primary Response*

Group	Log passive antiserum titre	Days of admin	Log_{10} 19S PFC per spleen ± SE	P*	Log_{10} 7S PFC per spleen ± SE	P*
I	0		4 245 ± 0 056		5 378 ± 0 056	
II	9	2	3 452 ± 0 082	<0 001	4 396 ± 0 096	<0 001
III	6	2	3 233 ± 0 043	<0 001	4 393 ± 0 058	<0 001
IV	3	2	3 512 ± 0 091	<0 001	4 142 ± 0 164	<0 001
V	9	1	3 252 ± 0 045	<0 001	<2 000§	<0 001
VI	6	1	3 373 ± 0 069	<0 001	3 758 ± 0 081	<0 001
VII	3	1	3 194 ± 0 112	<0 001	3 541 ± 0 120	<0 001

Six mice in each group

* P values from students t test by comparing the antiserum treated groups with the control group

§ Not significantly different from untreated control mice

TABLE 3 *Log₂ Haemagglutination Titre of the Humoral Antibody Response on Day 30 after Challenge*

Group	7S titre of passive antiserum	4×10^{1} SRBC dose		4×10^{9} SRBC dose	
		Total titre ± SE	7S titre ± SE	Total titre ± SE	7S titre ± SE
I	0	3 8 ± 0 5	2 5 ± 0 3	7 5 ± 0 2	6 4 ± 0 2
II	9	6 1 ± 0 2	3 5 ± 0 4	7 5 ± 0 3	7 4 ± 0 3
III	6	3 7 ± 0 2	1 8 ± 0 3	6 6 ± 0 2	5 4 ± 0 2
IV	3	1 5 ± 0 2	1 0 ± 0 2	6 5 ± 0 4	5 4 ± 0 5
V	9	4 7 ± 0 2	2 9 ± 0 1	5 5 ± 0 2	4 1 ± 0 3
VI	6	3 4 ± 0 5	1 0 ± 0 5	6 2 ± 0 3	4 7 ± 0 5
VII	3	4 5 ± 0 4	2 6 ± 0 6	6 1 ± 0 1	5 2 ± 0 2

Six mice in each group

in each group were immunized i v with 4×10^{6} SRBC and the other half with 4×10^{9} SRBC On day 30 after the primary antigen injection all groups received a second injection of 4×10^{8} SRBC

Group I received antigen only Groups II III and IV received decreasing amounts of passive antiserum on day 2 after antigenic challenge ($\log_2$ haemagglutination titres 9, 6 and 3, respectively) Groups V, VI and VII received the same quantities of antiserum on day 1 following challenge

The animals were tested on day 5 and day 30 in the primary response and on day 4 and day 7 in the secondary response by means of the haemolytic plaque assay

The mice were bled on day 30 in order to measure the anti SRBC haemagglutination titre before the secondary antigenic challenge

Table 1 shows the primary 19S and 7S cellular antibody responses of the groups challenged with the low antigen dose It can be seen that passive antiserum treatment on day 1 and day 2 after antigen was not able to suppress the 19S antibody response on day 5, the day of maximum response in this system (*Rubin* 1971a)

The 7S response (indirect plaques) was actually tested but either the mice did not respond at all or only very slightly

Table 2 shows the primary 19S and 7S cellular antibody responses on day 5 of the groups challenged with the high antigen dose It is evident that both the 19S and the 7S responses were reduced greatly by antiserum treatment Furthermore, antiserum treatment

TABLE 4 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10^{6} SRBC Primarily and 4×10^{8} SRBC Secondarily on the Number of PFC in the Spleen Tested on Day 4 in the Secondary Response*

Group	Log passive antiserum titre	Days of admin	$\log_{10}$ 19S PFC per spleen ± SE	P*	$\log_{10}$ 7S PFC per spleen ± SE	P*
I	0		4 878 ± 0 046		6 154 ± 0 079	
II	9	2	4 607 ± 0 120		4 737 ± 0 181	<0 001
III	6	2	4 264 ± 0 102	<0 001	4 529 ± 0 251	<0 001
IV	3	2	4 602 ± 0 045	<0 01	5 904 ± 0 053	
V	9	1	4 776 ± 0 092		<2 000§	<0.001
VI	6	1	3 520 ± 0 100	<0 001	4 813 ± 0 072	<0 001
VII	3	1	4 062 ± 0 051	<0 001	5 814 ± 0 050	<0 01

Six mice in each group

* As Table 2

§ Not significantly different from untreated control mice

TABLE 5 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10⁸ SRBC Primarily and 4×10⁸ SRBC Secondarily on the Number of PFC in the Spleen, Tested on Day 4 in the Secondary Response*

Group	Log₂ passive antiserum titre	Days of admin	Log_{10} 19S PFC per spleen ± SE	p*	Log_{10} 7S PFC per spleen ± SE	p*
I	0	–	4 606 ± 0 085		5 874 ± 0 038	
II	9	2	4 773 ± 0 200		5 605 ± 0 232	
III	6	2	4 335 ± 0 198		4 724 ± 0 145	
IV	3	2	4 740 ± 0 084		5 812 ± 0 058	
V	9	1	4 499 ± 0 049		<2 000§	<0 001
VI	6	1	3 979 ± 0 087	<0 005	5 386 ± 0 100	<0 005
VII	3	1	3 764 ± 0 091	<0 001	5 583 ± 0 081	

Six mice in each group
* As Table 2
§ Not significantly different from untreated control mice

on day 1 was much more effective in suppressing the 7S synthesis than antiserum treatment on day 2 whereas a similar difference in the suppressive effect on 19S antibody synthesis was not observed

The 19S and 7S cellular antibody responses of all groups of mice tested on day 30 following challenge were not significantly different from the background level of untreated control mice

The humoral antibody response on day 30 after primary antigenic challenge is shown in Table 3 It can be seen from the groups stimulated with the low antigen dose that a 19S antibody synthesis occurred both in the control and in the groups treated with antiserum This is in agreement with the results of the primary cellular 19S response where no inhibition was found Nothing can be said about the 7S response in these groups, except that a slight humoral 7S response could be registered in the control group The 7S humoral antibody titre of the antiserum treated groups may be due to the passively injected 7S antibody

The haemagglutination titre of the groups treated with antiserum stimulated with the high antigen dose shows a remarkable similarity to the humoral response of the control group treated with antigen, thus suggesting a well balanced regulation mechanism of the immunological apparatus in the treated mice

TABLE 6 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10⁸ SRBC Primarily and 4×10⁸ SRBC Secondarily on the Number of PFC in the Spleen Tested on Day 7 in the Secondary Response*

Group	Log passive antiserum titre	Days of admin	Log_{10} 19S PFC per spleen ± SE	p*	Log_{10} 7S PFC per spleen ± SE	p*
I	0		2 901 ± 0 110		4 275 ± 0 020	
II	9	2	3 586 ± 0 139	<0 01	4 283 ± 0 168	
III	6	2	3 426 ± 0 064	<0 02	3 844 ± 0 141	
IV	3	2	3 516 ± 0 048	<0 001	4 368 ± 0 058	
V	9	1	3 165 ± 0 059		3 154 ± 0 052	<0 001
VI	6	1	3 538 ± 0 094	<0 005	3 972 ± 0 067	<0 005
VII	3	1	2 869 ± 0 093		3 700 ± 0 065	<0 001

Six mice in each group
* As Table 2

TABLE 7 *Effect of Passive Antiserum Given 1 or 2 Days after Administration of 4×10[?] SRBC Primarily and 4×10[?] SRBC Secondarily on the Number of PFC in the Spleen, Tested on Day 7 in the Secondary Response*

Group	Log passive antiserum titre	Days of admin	Log_{10} 19S PFC per spleen ± SE	P*	Log_{10} 7S PFC per spleen ± SE	P*
I	0	–	2 502 ± 0 154		4 198 ± 0 109	
II	9	2	3 336 ± 0 107	<0 005	4 092 ± 0 117	
III	6	2	2.572 ± 0 096		3 918 ± 0 034	
IV	3	2	3 128 ± 0 100		3 881 ± 0 056	
V	9	1	2 795 ± 0 045		3 578 ± 0 062	<0 005
VI	6	1	2 952 ± 0 085		3 624 ± 0 123	<0 01
VII	3	1	2.591 ± 0 167		3 658 ± 0 078	<0 01

Six mice in each group
* As Table 2

All groups of mice were rechallenged with 4×10[5] SRBC on day 30 Table 4 shows the secondary 19S and 7S cellular antibody responses of the groups which received the low primary antigen injection The test was performed on day 4 (the day of maximum secondary response) It can be seen that the 7S synthesis was strongly suppressed (in one case completely inhibited group V) with the high and medium quantity of antiserum whereas the low dose of antiserum in groups IV and VII reduced the 7S response only slightly

Correspondingly it was found that the 19S synthesis was reduced only in the groups treated with the medium and low concentrations of passive antiserum The lack of inhibition in groups II and V suggests the existence of a 7S feedback inhibition on 19S synthesis since these groups were those in which the 7S inhibition was most pronounced

Table 4 also shows that antiserum treatment on day 1 gave a much more pronounced inhibition of both 19S and 7S syntheses than antiserum treatment on day 2

Table 5 shows the secondary response of the groups challenged with the high primary antigen dose It can be seen that suppression (and in one case even complete inhibition) was obtained in both 19S and 7S antibody responses but the suppression was relatively less pronounced with the high primary antigen dose than with the low dose It is characteristic that inhibition was obtained only in those groups that received antiserum on day 1 after antigen The absence of 7S feed back inhibition on the 19S synthesis mentioned above can also be seen from these results

Tables 6 and 7 show the cellular antibody production on day 7 in the secondary response at which time the decay of the antibody synthesis at the cellular level has begun The 7S synthesis was still suppressed but only in the groups that received antiserum on day 1

On the other hand, the 19S synthesis in groups II III, IV and VI in Table 6 and group II in Table 7 shows an enhancement phenomenon as compared to group I In the light of the previous inhibition of 7S antibody this finding is perhaps the best and most pronounced evidence for a 7S regulating effect on the 19S synthesis

DISCUSSION

The present experiments were carried out to elucidate the effect of passive antiserum treatment on the immunological memory

Using a low and a high antigen dose for the primary response and antiserum treatment in different quantities on days 1 and 2 after antigenic stimulation the results of *Moller* (1969) were confirmed *viz* passive

antibody suppresses the primary immune response to a high antigen dose selectively

This means that the low dose of antigen stimulates the immune apparatus to a normal primary response within day 1, whereas both the 19S and 7S antibody syntheses elicited with a *high antigen dose are dependent on* the presence of antigen even after day 2

These findings are in agreement with the conception of "cell selection by antigen" hypothesis (see introduction) The low antigen dose stimulates mainly antigen-sensitive cells with receptors of relatively high affinity None of the amounts of passive antiserum used were able to interfere with this antigen stimulation when given on day 1 or day 2 The high antigen dose stimulates cells with receptors representing a wider range of affinities possibly causing cells with high affinity receptors to become tolerant (*Theis* & *Siskind* 1968) Thus in this situation passive antiserum can compete effectively with the antigen sensitive cells that are less efficient

The finding that the immune response to a high antigen dose is more dependent on the continuous presence of antigen may be explained by the assumption that antigen stimulation of cells with low and medium affinity receptors is accomplished more slowly than that of cells with high affinity receptors

When mice challenged with the low antigen dose were *rechallenged with* 4×10^{8} SRBC on day 30 only a weak 19S memory response (calculated as net excess secondary response (*Nossal et al* 1965)) was observed, whereas the 7S memory response was very high (see also *Rubin* 1971b)

However treatment with passive antiserum did not raise a 19S memory response though the 7S memory response was depressed in linear proportion to the dose of passive antiserum

When mice challenged with the high antigen dose were rechallenged with 4×10^{8} SRBC on day 30 a secondary 19S PFC response equal to or lower than the maximum primary response (on day 4 (*Rubin* 1971b)) was observed The 7S memory response was very high, although lower than the memory response obtained when using the low/high dose combination In the same way as in this dose combination treatment with passive antiserum did not raise a 19S memory response though the 7S memory response was depressed However, the depression of the 7S *memory response was generally lower in this* high/high dose combination than in the low/high dose combination

In a previous report in this series (*Rubin* 1971b), it was concluded that the absence of 19S memory was due to an inhibitory activity of the endogenously produced 7S antibody on the 19S memory cells The results presented here suggest that this kind of inhibition can not be the only factor responsible, since no 19S memory developed when the primary 7S response elicited by the high antigen dose was suppressed with passive antiserum It may thus be concluded that 19S memory cells elicited by a high antigen dose are rendered tolerant (mediated by antibody (*Diener* & *Feldmann* 1970, *Feldmann* & *Diener* 1970)) or develop into 7S PFC and 7S memory cells (according to the XYZ scheme of immunocyte maturation (*Sercarz* & *Byers* 1967, *Sterzl* 1967))

The conception that a 7S feedback mechanism reported by various investigators (*Sahiar* & *Schwartz* *1964*, *Möller* & *Wigzell* 1965 *Henry* & *Jerne* 1967) is an im*portant regulation mechanism can be seen on* day 7 after restimulation in groups where the 7S synthesis was suppressed Here the 19S antibody synthesis was enchanced as compared to the control groups treated with antigen only

However it should be emphasized that when the regulation of antibody synthesis is being studied doses of antigen lower than those used in the present study should be preferred This applies especially when using complex antigens like SRBC It is a clear cut corollary of the "cell selection by antigen" hypothesis (*Siskind* & *Benacerraf* 1969) that a low dose of antigen stimulates only a limited number of cells all of which probably carry high affinity receptors Since these cells presumably proliferate and differentiate

simultaneously, the restimulation of their daughter cells with a low antigen dose should give more conclusive results

The authors wish to thank Miss *E Nielsen* for excellent technical assistance

We are also grateful to Mr *F Jacobsen* for his care of the animals, and to Mr *F Valsted* and Mr *W Petersen* for performing the injections

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UTILIZATION OF THYMINE, THYMIDINE AND TMP BY *NEISSERIA MENINGITIDIS*

2 *Lack of Enzymes for Specific Incorporation of Exogenous Thymine, Thymidine and TMP into DNA*

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Activities corresponding to thymidine phosphorylase and nucleoside deoxyribosyl transferase could not be found in *Neisseria meningitidis* ^{14}C 2 thymine was bound to or taken up by whole cells but this did not result in labelling of the TMP TDP or TTP pools No significant thymidine kinase activity could be induced in *N meningitidis* ^{14}C 2 thymidine which was bound to or taken up by whole cells resulted in faint labelling of the TMP TDP and TTP pools after prolonged incubation but no radioactive thymine appeared The *N meningitidis* extracts had activities corresponding to TMP and TDP kinases but whole cells which were exposed to TMP methyl ^{3}H did not seem to take up significant amounts of this compound The findings indicate that *N meningitidis* lacks the enzyme systems that are known to mediate a specific incorporation of thymine or thymidine into DNA

The lack of thymine thymidine or TMP deficient mutants in *Neisseria meningitidis* and the wish to label DNA specifically for genetic experiments led to the study of uptake of these compounds when exogenously added (15) The growth rate was slightly increased upon their addition to cultures of meningococci and at the same time radioactivity was taken up from labelled thymine to an extent of 5 6 per cent of the material added from thymidine to an extent of 0 6 per cent and from TMP to an extent of 0 05 per cent

The uptake in *N meningitidis* of radioactivity from thymine or thymidine could not be increased by the addition of deoxyribonucleosides (15) in the way it has been described for *Escherichia coli* (21) and *Haemophilus influenzae* (8) The finding that the base analogue 5 fluorodeoxyuridine does not increase the uptake of radioactivity from thymine or thymidine further emphasizes that the situation in *N meningitidis* (15) is different from that in *Haemophilus* (28)

In the present work *N meningitidis* has been examined for the presence of enzyme functions known to be involved in the utilization of thymine or thymidine The overall scheme for specific incorporation of exogenous thymine thymidine or TMP into DNA may be described as follows

Thymine $\xrightarrow{1}$ thymidine $\xrightarrow{2}$ TMP $\xrightarrow{3}$ TDP $\xrightarrow{4}$ TTP $\xrightarrow{5}$ DNA

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1a (catabolism) Thymidine phosphorylase, EC 2 4 2 4 (2, 3)
thymidine + P_i = thymine + deoxyribose–1–P

1b (anabolism) Nucleoside deoxyribosyl transferase, EC 2 4 2 6 (2)
thymidine + base = thymine + deoxyribose–base

2 Thymidine kinase, EC 2 7 1 21 (2)
thymidine + ATP = TMP + ADP

3–4 TMP and TDP kinases, EC 2 7 4 4 and EC 2 7 4 6 (2)
TMP + ATP = TDP + ADP
TDP + ATP = TTP + ADP

5 DNA polymerase

MATERIALS AND METHODS

The media and methodology employed in this investigation were analogous to those previously used (15) The bacterial strains were also the same, with the *N meningitidis* strain M1 as the main test microbe In addition to the other strains, a strain belonging to Group A was also used (Strain 2686) In most experiments the meningococcal strain under study was compared with an *E coli* K 12 strain (15)

Cell free extracts The cells were harvested from the surface of solid media in saline, washed with saline and suspended in the buffer to be used for the actual experiment In some experiments the cells were grown in fluid complete medium or in the basal media (15), harvested by centrifugation washed with saline and suspended as described The suspension was cooled in a salt ice water bath and treated at maximum oscillation in a M S E ultrasonic disintegrator (60 watts) for three minutes with intermediate cooling (1½ min × 2) The cell debris was removed by centrifugation at 7900 × g for 30 min, and the supernatant was examined for enzyme activities Protein was determined by the procedure of *Lowry et al* (20)

Thymidine phosphorylase EC 2 4 2 4 (2) This enzyme was assayed for by analysis of thymine synthesis from thymidine as described by *Kammen* (16) Crude extracts (0 1 0 2 ml) of *N meningitidis* or *E coli* in Tris HCl buffer pH 7 2 were mixed with 160 μmoles sodium phosphate pH 6 1 7 0 or 8 0 and 2 mg of thymidine in a total volume of 1 1 ml Aliquots (0 2 ml) were removed after various periods (usually ½ 5, 15, 30 and 40 min) of incubation at 37°C and were mixed with 2 4 ml of 0 3 M NaOH Appropriate controls lacking enzyme or containing Tris HCl buffer instead of phosphate were included Phosphorolysis of thymidine was determined from the change of absorbance at 300 mμ Thymidine phosphorylase was also assayed by measurement of deoxyribose synthesis according to the method of *Munch Petersen* (21) Unless otherwise noted, the reaction mixture contained in a total volume of 2 5 ml 150 μmoles sodium phosphate pH 6 1, 6 8 or 7 0, 2 5 mg thymidine (10 3 μmoles) and crude bacterial extract or a suspension of bacteria Incubation times and controls were as in the photometric assay At the end of the experiment samples (usually 650 μl) were pipetted into 50 μl 70 per cent perchloric acid with an additional 100 μl of 0 5 N $HClO_4$ Deoxyribose was determined according to *Burton* (7)

Nucleoside deoxyribosyl transferase, EC 2 4 2 6 (2) was also assayed by the method of *Munch Petersen* (21) The system contained in a total volume of 2 5 ml 100 μmoles Tris HCl buffer pH 7 2 2 5 mg thymidine, 0 3 mg adenine (2 2 μmoles) and crude bacterial extract or a suspension of bacteria Incubation removal of samples and analysis of deoxyribose were as described for the thymidine phosphorylase assay

Thymidine kinase, EC 2 7 1 21 (2) Unless otherwise noted this enzyme activity was assayed in a total volume of 190 μl by mixing 10 μmoles Tris HCl buffer pH 8 0, 1 μmole ATP, 1 μmole $MgCl_2$, 1 μmole 3 phosphoglycerate, 0 018 μmole (54 5 μc/μmole) ^{14}C 2 thymidine and 50 μl crude extract Incubation was at 37°C for 5, 10, 15 30 and 60 min The reaction was stopped by cooling Protein was precipitated by the addition of 20 μl 100 per cent (w/v) TCA and removed by centrifugation (4) Analysis was performed by two chromatographic procedures

Method I 40 μl of the clear supernatant was applied to Whatman No 1 paper for chromatography in the isobutyric acid solvent of *Krebs & Hems* (18) Carrier thymidine, TMP, TDP and TTP (20–30 μg of each) were spotted The positions of the nucleotides on the chromatograms were located with ultraviolet light and marked in pencil The radioactivity was located by strip chart recording The relative activities of the spots were determined by planimetry and calculated in terms of ^{14}C by means of spots from the ^{14}C 2 thymidine originally added to the reaction mixture (14) *Method II* In some experiments the chromatograms were run in two dimensions with the following solvents the first was the isobutyric acid solvent (18), and the second the n butanol acetone acetic acid conc NH_3 H_2O solvent as described below (12, 23) The location of the radioactivity was determined by autoradiography

TMP kinase EC 2 7 4 4 (2) A reaction volume of 115 μl consisted of 5 μmoles Tris HCl buffer

TABLE 2 *Search for Thymidine Posphorylase and Nucleoside Deoxyribosyl Transferase in N meningitidis Variations in Experimental Conditions*

Expt series no	Condition examined	Variations performed
1	Growth phase of the bacteria	Harvested from early log phase late log phase and from stationary phase
2	Medium used	Growth on Medium A Medium KC and complete medium
3	Induction	Cells grown in the presence of thymidine compared with cells grown without
4	Treatment of the cells	Measurements performed in sonic extracts and in whole cells
5	Buffer system used during the assay	Thymidine phosphorylase examined with phosphat buffer pH 6 1, 6 8 and 7 0 Nucleoside transferase with Tris HCl buffer pH 7 2 and with phosphate buffer pH 6 1
6	Organism tested	Search for activities performed in strains belonging to Group A Group B and Group C

TABLE 3 *Search for Thymidine Kinase Activity in Cell free Extracts from N meningitidis an E coli*

Expt no	Extract used	Protein mg/assay	Time of incubation min	Incorporation calculated as mμmoles thymidine		
				TMP	TDP	TTP
1	*N meningitidis*					
	Strain M1*	0 24	5			
	Strain M1*	0 24	10			
	Strain M1*	0 24	30		–	–
	Strain M1*	0 24	60			
2	*E coli*	0 44	5	1 26		
	E coli	0 44	15	3 09	0 34	0 50
3	*E coli*	0 22	60	1 06	0 18	0 29
	E coli + *N meningitidis* Strain M1	0 08	60	0 33	0 39	0 29
	E coli + *N meningitidis* Strain M6	0 12	60	0 82	0 38	0 15

Assay as described in Methods with analysis by chromatography strip chart recording and plani metry (Method I)

* Strain M6 Strain M5 and Strain 2686 gave the same results

the cells on basal media in the presence of thymidine In contrast *E coli* extracts showed high activity with pronounced incorporation of radioactivity from ^{14}C–2 thymidine into TMP, TDP and TTP (Table 3) The distribution of incorporation in the three nucleotides points to an active TMP and TDP kinase in the *E coli* extracts The lack of thymidine kinase activity in the *N meningitidis* extracts might conceivably be due to

TABLE 4 *Incorporation of Radioactivity from ¹⁴C 2 thymidine into TMP, TDP and TTP in the Presence of Extracts from* N meningitidis *and* E coli

Organism	Age of extract	Protein mg/assay	Time of incubation min	Incorporation calculated as μμmoles thymidine		
				TMP	TDP	TTP
N meningitidis						
Strain M1	New	0 20	30	4 8	1 7	3 9
Strain M1	New	0 20	60	3 9	1 5	2 3
E coli	8 months	0 24	30	98 0	3 8	1 8

Experimental system as for the thymidine kinase assay Analysis by chromatography in to solvents (Method II)

inhibitors, or to factors that destroy the enzyme Therefore, cellfree extracts of *E coli* were mixed with *N meningitidis* extracts and incubated at 37°C for 30 min prior to the assay for kinase activity The activities were found to be the same as in the *E coli* extracts alone when the activity was calculated as the sum of the incorporation in TMP, TDP and TTP But these experiments show that the addition of *N meningitidis* extracts results in some change in the distribution of radioactivity between the nucleotides This finding was taken to indicate that *N meningitidis* extracts contain nucleoside phosphate kinases, an assumption which was confirmed by the assay with ³H TMP (described below)

When the thymidine kinase assay system was analysed by Method II, chromatography in two solvents and autoradiography there was a pronounced radioactive spot on the film corresponding to TMP, and faint spots on the film corresponding to TDP and TTP when *E coli* extracts were used, even when this extract had been kept frozen at 20°C for eight months With extracts from *N meningitidis* (Strain M1) there were faint spots on the film corresponding to TMP, TDP and TTP after prolonged incubation (Table 4)

TMP and TDP kinases Crude extracts of *N meningitidis* effectively converted TMP into TDP and TTP as shown in Table 5

Reactions in Intact Cells

The metabolism of ¹⁴C–2–thymine, ¹⁴C–2–thymidine and TMP–methyl–³H added to cultures of *N meningitidis* was next investigated by measuring the transfer of radioactivity to various intermediates, particularly to the intracellular pools of TMP, TDP and TTP Again *E coli* K12 was simultaneously examined to provide a means for comparison with better known systems (2, 12, 21)

Labelling from ¹⁴C–2–thymine Previous experiments showed that *N meningitidis* cells are labelled from ³H–thymine (15) In the present experiments with ¹⁴C–2 thymine no

TABLE 5 *TMP and TDP Kinase Activity in Cell free Extracts from* N meningitidis

Organism	Protein mg/assay	Time of incubation min	³H TMP recovered mμmoles	Incorporation calculated as mμmoles ³H TMP	
				³H TDP	³H TTP
N meningitidis					
Strain M1	0 20	30	1 989	0 094	0 052
Strain M1	0 20	60	1 972	0 154	0 107

TABLE 6 *Labelling of Thymine and Adenine Nucleotides from ^{14}C-2-thymine and ^{14}C-8-adenine in Intact Cells*

Expt no	Organism	Labelled material added	Deoxy-nucleoside added	Time of incubation	Incorporation calculated as μμmoles thymine or adenine							
					Thymidine	TMP	TDP	TTP	Adenosine	AMP	ADP	ATP
	N. meningitidis	^{14}C thymine										
1	Strain M6	2 μg/ml	None	2 min*		–	–			–	–	–
	Strain Mo	2 μg/ml	AdR**	2 min*				–		–	–	–
2	*E. coli*	2 μg/ml	None	2 min*		–	–	–	–	–	–	–
	E. coli	2 μg/ml	AdR	1 min	29.1	17.4	91.4	102.0	–	–	–	–
	E. coli	2 μg/ml	UdR	2 min	80.5	0.6	10.5	10.5	–	–	–	–
	E. coli	2 μg/ml	CdR	2 min	101.0	1.2	16.2	9.4		–	–	–
	N. meningitidis	^{14}C adenine										
3	Strain Mo	2.6 μg/ml	None	15 sec			–	–		135.0	217.0	221.0
	Strain M6	2.6 μg/ml	None	30 sec		–	–	–	–	318.0	361.0	313.0

* Other times tested as shown in Methods

** Also tested GdR, IdR, UdR and CdR

TABLE 7 *Labelling of TMP, TDP and TTP from ¹⁴C 2 thymidine in Intact Cells*

Organism	Thymidine µg/ml	Deoxynucleoside added	Time of incubation min	Incorporation calculated as µµmoles thymidine				
				Thymine	Thymidine	TMP	TDP	TTP
N meningitidis								
Strain M1	2 2	None	5	–	0 82	1 4	1 3	0 8
Strain M1	2 2	None	15	–	0 45	2 0	2 5	0 8
E coli	2 2	None	2	300 0	Not measured	6 5	101 6	69 4
E coli	2 2	AdR	1	8 9	Not measured	24 4	453 0	47 9

radioactivity appeared in thymidine, TMP, TDP or TTP (Table 6) Also, no significant pool of labelled thymine could be demonstrated in the cells after exposure to ¹⁴C–2–thymine Since it is known that *E coli* strains without thymine requirement do not normally incorporate radioactive thymine into DNA, addition of deoxyribonucleosides during the assay was also attempted (21, 22) Even under these conditions no incorporation in the same intermediates was observed Examination of other strains of *N meningitidis* gave the same results Control experiments with *E coli* showed rapid incorporation of radioactivity from thymine in the presence of deoxyribonucleosides

In contrast to thymine, adenine is readily incorporated into *N meningitidis* cells with intense spots corresponding to AMP, ADP and ATP already after a few seconds (Table 6) It is of considerable interest that no label from adenine appeared in adenosine indicating that the incorporation could be via an adenine phosphoribosyl transferase, EC 2 4 2 7 (2, 26) Attempts to demonstrate a thymine phosphoribosyl transferase were entirely negative

Labelling from ¹⁴C–2–thymidine Thymidine was obviously taken up or bound to the surface of meningococci to some extent In contrast to the thymine experiments, some labelling also occured of TMP, TDP and TTP (Table 7) But the incorporation is very weak compared to that obtained in the experiments with *E coli*, and the radioactivity appeared after a long time of incubation No break down of thymidine to thymine could be observed in the *N meningitidis* cells, and

TABLE 8 *Labelling of TDP and TTP from ³H TMP in Intact Cells*

Organism	TMP* added µg/ml	Time of incubation min	Incorporation calculated as µµmoles TMP				
			Thymine	Thymidine	TMP	TDP	TTP
N meningitidis							
Strain M1	6 7	5	0 34	0 02	4 0	9 7	2 3
Strain M1	6 7	15	–	0 34	3 9	8 0	5 3
E coli	6 7	5	51 0	46 0	Not measured	160 0	75 0

* Analysis of the ³H TMP preparation by chromatography showed the following composition Thymine 0 84 per cent thymidine 6 80 per cent and TMP 92 86 per cent

TABLE 6 *Labelling of Thymine and Adenine Nucleotides from ^{14}C 2 thymine and ^{14}C 8 adenine in Intact Cells*

Expt no	Organism	Labelled material added	Deoxy nucleoside added	Time of incubation	Incorporation calculated as µµmoles thymine or adenine							
					Thymidine	TMP	TDP	TTP	Adenosine	AMP	ADP	ATP
	N meningitidis	^{14}C thymine										
1	Strain M6	2 µg/ml	None	2 min*							—	—
	Strain M6	2 µg/ml	AdR**	2 min*						—	—	—
	E coli	2 µg/ml	None	2 min*			—	—			—	—
2	*E coli*	2 µg/ml	AdR	1 min	29 1	17 4	94 4	102 0		—	—	—
	E coli	2 µg/ml	UdR	2 min	80 5	0 6	10 5	10 5		—	—	—
	E coli	2 µg/ml	CdR	2 min	101 9	1 2	16 2	9 4		—	—	—
	N meningitidis	^{14}C adenine										
3	Strain M6	2 6 µg/ml	None	15 sec			—	—		135 0	217 0	221 0
	Strain M6	2 6 µg/ml	None	30 sec		—	—	—	—	318 0	361 0	313 0

* Other times tested as shown in Methods
** Also tested GdR IdR UdR and CdR

TABLE 7 *Labelling of TMP, TDP and TTP from ¹⁴C 2 thymidine in Intact Cells*

Organism	Thymidine µg/ml	Deoxy nucleoside added	Time of incubation min	Incorporation calculated as µµmoles thymidine				
				Thymine	Thymidine	TMP	TDP	TTP
N meningitidis								
Strain M1	2.2	None	5	–	0.82	1.4	1.3	0.8
Strain M1	2.2	None	15	–	0.45	2.0	2.5	0.8
E coli	2.2	None	2	300.0	Not measured	6.5	101.6	69.4
E coli	2.2	AdR	1	8.9	Not measured	24.4	453.0	47.9

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TABLE 8 *Labelling of TDP and TTP from ³H TMP in Intact Cells*

Organism	TMP* added µg/ml	Time of incubation min	Incorporation calculated as µµmoles TMP				
			Thymine	Thymidine	TMP	TDP	TTP
N meningitidis							
Strain M1	6.7	5	0.34	0.02	4.0	9.7	2.3
Strain M1	6.7	15		0.34	3.9	8.0	5.3
E coli	6.7	5	51.0	46.0	Not measured	160.0	75.0

* Analysis of the ³H TMP preparation by chromatography showed the following composition Thymine 0.84 per cent thymidine 6.80 per cent and TMP 92.86 per cent

the addition of deoxyadenosine did not increase the labelling of the intermediates measured

Labelling from ^3H–TMP The incubation of *N meningitidis* cells with ^3H–TMP resulted in a labelling of the TMP, TDP and TTP pools which is extremely faint compared to that found in the control experiments with *E coli* cells (Table 8) The incorporation found in the nucleotide pools is comperable to that found in experiments with uptake from ^{14}C–thymidine (Table 7) Analyses of the ^3H–TMP used also showed that it was contaminated with 6 8 per cent thymidine If 0 6 per cent of this thymidine is taken up (15) the contaminating thymidine may account for all the label found in TMP, TDP and TTP

In E coli cells TMP is obviously taken up and broken down to thymidine and thymine resulting in a significant labelling of these compounds (Table 8) In contrast the labelling corresponding to thymine and thymidine which is still observed in the *N meningitidis* cells after repeated washing is very faint and may represent contaminating thymine and thymidine which is taken up or bound to the cells

DISCUSSION

These experiments indicate that *N meningitidis* lacks the enzyme thymidine phosphorylase and nucleoside deoxyribosyltransferase and that no direct labelling of TMP TDP or TTP can take place from radioactive thymine The negative results with thymine are emphasized by the uptake of radioactivity into intact cells from labelled adenine which is very pronounced after a few second and mediates a heavy labelling of AMP ADP and ATP Since the control experiments with adenine indicate that the incorporation of adenine does not occur via adenosine and may be mediated by an adenine phosphoribosyl transferase attempts were also made to demonstrate such an activity with thymine as a substrate even if such activity does not seem to be known from other systems (2 26) These results were altogether negative

Exogenous radioactive thymine does indeed result in some labelling of *N meningitidis* cells (15 17), but the present experiments indicate that it is not incorporated directly into TMP, TDP or TTP Therefore we must assume that the substance is broken down before it is metabolized As a consequence uptake of thymine will probably not result in a specific labelling of DNA in *N meningitidis*

No significant thymidine kinase activity could be induced in *N meningitidis* by means of growth in the presence of thymidine although radioactivity is taken up from labelled thymidine to an extent of 0 6 per cent (15), and experiments with intact cells showed that exogenous thymidine is taken up by or bound to the cells to some extent When employing a very sensitive technique it was indeed found that some radioactivity appeared in TMP, TDP and TTP after incubation of *N meningitidis* extracts as well as cells with radioactive thymidine But the activity appeared late and amounted to at most one per cent of that found in *E coli* cells Even an 8 months old extract of *E coli* caused an incorporation in the nucleotides more than ten times higher than that found with fresh *N meningitidis* extracts under otherwise identical conditions If this incorporation in *N meningitidis* indicates the existence of a thymidine kinase the activity must be extremely low compared to that of *E coli* It may thus be that the observed incorporation of radioactivity from thymidine into the nucleotides of *N meningitidis* represents some other mechanism than a thymidine kinase such as discussed in connection with thymidine uptake in some microorganisms as well as plants and mammalian cells (1 5 10)

The extracts from *N meningitidis* had pronounced activities corresponding to TMP and TDP kinases It is of interest that crypticism was observed in connection with these activities in the sense that they could

not be reproduced with intact cells But the experiments with intact cells certainly indicated a very faint incorporation of exogenous ^{3}H-TMP into the TMP, TDP and TTP pools However the calculations indicate that this may be accounted for by the uptake of radioactive thymidine which contaminated the ^{3}H-TMP used In agreement with previous findings (15) it may thus be assumed that intact TMP is not taken up by *N meningitidis* cells to any significant extent This finding may explain why all attempts to select mutants requiring TMP have been negative (15)

The control experiments concerned with ^{3}H TMP uptake in *E coli* are in agreement with the findings of *Breitman et al* (6) that TMP is taken up by *E coli* cells, and is probably specifically incorporated into DNA

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PRODUCTION OF PHOSPHOLIPASE C IN *ACINETOBACTER CALCOACETICUS*

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The production of phospholipase C (haemolysin) of *Acinetobacter calcoaceticus* in a chemically defined medium is described. A molecular weight of the enzyme of 25–35,000, and an isoelectric point of 6.75 were estimated by gel filtration and isoelectric focusing experiments.

In preceding papers (6, 7), the haemolytic and phospholipase activities of *Acinetobacter calcoaceticus* were described. The findings indicated that the haemolytic activity was due to a phospholipase C.

Phospholipase C was previously estimated by various nonspecific methods. Methods employing purified lecithin substrates are now available. Lecithin sols are obtained by means of ultrasonic dispersion or anionic detergents. The reaction rate is determined by estimating the phosphorus liberated from phosphorylcholine by wet ashing or enzymatic hydrolysis. (For surveys see *Kurioka & Liu* (5), *Oakley* (8).)

The aim of this paper is to test a chemically defined, low molecular weight medium for production of *A. calcoaceticus* haemolysin, to give preliminary data of the molecular size and charge of the haemolysin, and to demonstrate the presence of phospholipase C.

Received 7.vi.71

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MATERIALS AND METHODS

The strain of *A. calcoaceticus* used (1318/69), methods for estimation of growth rate, haemolytic activity and acid soluble phosphorus, as well as the method of extraction of lipids, are described in previous papers (6, 7).

Chemicals. L-α lecithin (ex egg, grade I) and L-1,2 diglyceride were obtained from Koch Light Lab., Colnbrook, Bucks., England. Phosphorylcholine was purchased from Sigma Chem. Comp., St. Louis, Missouri, USA. Carrier ampholytes were obtained from LKB Produkter AB, Bromma, Sweden. Reference proteins (Protein Calibration Kit) were supplied by Boehringer, Mannheim GmbH, Germany. All other chemicals used were analytical grade.

Culture medium. This was prepared according to Joner (personal communication) and contained (mmol/l): NH_4Cl 19, KH_2PO_4 26, Na_2HPO_4 51, NaCl 10, xylose 20, asparagine 24, $MgSO_4$ 1, $CaCl_2$ 0.1 and $FeCl_2$ 0.001. Roux bottles containing 100 ml of medium were inoculated with 1 ml of a 48 hrs culture of the organism and incubated aerobically at 37° C. Growth rate and haemolytic activity were estimated at intervals of 24 hrs.

Preparation of crude filtrate. After 72 hrs, the cultures were centrifuged in a Servall Superspeed centrifuge (10 000 g, 4° C, 90 min), filtered through a Millipore membrane (HAWP, 0.45 µ) and concentrated (10 times by volume, at 4° C) in an Amicon Ultrafiltration cell (Lexington, Mass., USA), using a Diaflo Ultrafiltration mem-

brane (UM 20E, diam 42 mm, pressure 3 atm N_2) Finally, the filtrate was dialyzed for 24 hrs at 5 ± 1° C against running tap water and stored at —20° C until use

Molecular weight determination Sephadex G 200 was equilibrated with 0 05 M tris pH 7 6, containing 0 13 M NaCl, and calibrated with reference proteins of known molecular weights Calculations were performed as described by *Andrews* (1)

Isoelectric focusing This was performed in an LKB 8101 Ampholine Column (110 ml) according to *Vesterberg & Svensson* (9), employing 1 per cent (w/v) ampholytes, pH range 3 10 dissolved in a 0–50 per cent (w/v) sucrose gradient Crude filtrate (600 H U) was added to the less dense solution Potentials varied between 350–600 V, the experiment lasting for 45 hrs at 4° C Elution was carried out in 2 5 ml fractions, pH was measured at 22° C in a Methrom pH meter, followed by determination of haemolytic activity, acid soluble phosphorus and protein (optical density at 280 nm in a Beckman DBG-spectrophotometer)

Assays for enzymatic activity A solution of 15 mM (in some experiments mentioned later 25 mM) lecithin in 0 2 M tris pH 8 containing 0 29 M NaCl and 0 02 M $MgCl_2$ was treated with a Mullard Ultrasonic Disintegrator at 20 kc/s for 10 min One part lecithin sol and 3 parts crude filtrate were mixed and incubated at 37° C Samples of 3 ml were taken at the start and after 5 hrs incubation precipitated with 0 6 ml 30 per cent (w/v) trichloracetic acid and filtered through a filter paper The supernatant fluid was used for estimation of acid soluble phosphorus and for chromatography of phosphorylcholine Lipids in the precipitate were extracted to a final volume of 0 5 ml chloroform methanol (2 1, v/v)

Thin layer chromatography of lipids TLC was carried out on silica gel plates, 20×20 cm, thickness 0 25 mm activated for 2 hrs at 110° C The developing system for diglycerides was light petroleum-ethyl acetate (75 25, v/v) Separated lipids were visualized by charring at 110° C after spraying with 9 M H_2SO_4 Phospholipids were detected by means of a molybdenum spray (3)

Paper chromatogrphy og phosphorylcholine Descending chromatography was carried out on Selecta filter paper (no 2043b, Mgl 23×45 cm), in a Shandon 300 Chromotank employing butanol acetic acid water (60 15 25, v/v/v) The spots

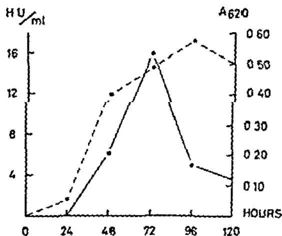


Fig 1 Growth Curve and Haemolytic Activity 5 ml samples of the culture were centrifuged in a Servall Superspeed centrifuge and the haemolytic activity (———) was examined in the supernatant fluid Turbidity of the culture (- -) was read in a Linson III photometer at 620 nm

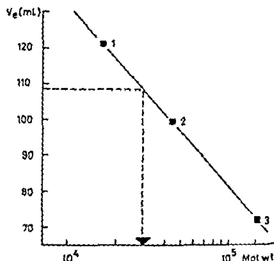


Fig 2 Molecular Weight Determination of Haemolysin by Gel Filtration Sephadex G 200 was allowed to swell for 7 days in the elution buffer (0 05 tris pH 7 6 containing 0 13 M NaCl) and was then deaerated and filled onto a column (35 cm × 2 5 cm diam , bed volume ~160 ml) supplied with a water jacket (5±1° C) The column was equilibrated for 24 hrs (pressure 15 cm H_2O flow rate 4 0 ml/cm²/hr) A sample solution 3 ml) containing proteins (5 mg) and 35 haemolytic units was applied to the top of the column by layering under the buffer solution Column effluents were collected in 2 5 ml portions in an LKB fraction collector Proteins were estimated by light extinction measurements at 280 nm (cytochrome C 420 nm) in a Beckman DBG spectrophotometer Haemolytic activity was measured according to the test method (6) Elution volume Ve) was plotted against log (mol wt) 1 cytochrome C 2 ovalbumin 3 aldolase Arrow indicates haemolytic activity

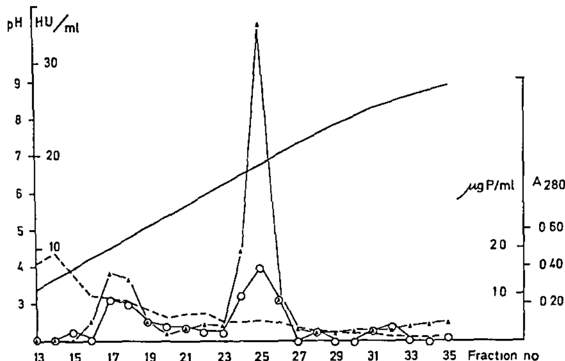


Fig 3 Isoelectric Focusing of Crude Filtrate See text for experimental details (———) pH ( ) protein (—▲—) haemolytic activity (—○—) acid soluble phosphorus liberated in 4 hrs in a reaction mixture consisting of a 1 5 ml portion from each fraction and 0 5 ml 25 mM lecithin sol dissolved in 0 2 M tris pH 8 containing 0 5 M NaCl and 0 0? M $MgCl_2$

were rendered visible according to the method of *Hanes & Isherwood* (4)

RESULTS

Haemolytic activity was not detected in cultures grown in agitated Erlenmeyer flasks Cultures in Roux bottles grown at 37° C (Fig 1) produced higher haemolytic titers than cultures grown at 25° C In the absence of asparagine haemolysin production was negligible and lower values were also obtained in cultures devoid of magnesium and in cultures where xylose was replaced by glucose

The haemolytic activity eluted on Sephadex G 200 corresponded to a molecular weight higher than cytochrome C (16 000) and lower than ovalbumin (45 000) in the region of 25 35 000 (Fig 2)

Isoelectric focusing (Fig 3) revealed a main fraction with maximum haemolytic activity at pH 6 75 and a small fraction at pH 4 5–4 75 Peak values of acid soluble phosphorus (liberated from lecithin) corresponding to both fractions were found

Lecithin (remaining at the point of application on TLC) was split into substances with mobilities identical to 1 2 diglyceride (Fig 4a) and phosphorylcholine (Fig 4b) During the incubation acid soluble phosphorus increased from 2 0 to 73 8 μg/ml

In a similar reaction mixture (with the exception that 25 nM lecithin sol was employed) the presence of 5 mmol/1 of MgCl increased the reaction rate by 66 per cent and 1 per cent (w/v) sodium deoxycholate increased the reaction rate by 53 per cent To obtain a one step procedure, the simultaneous splitting of liberated phosphorylcholine by alkaline phosphatase according to *Kurioka & Liu* (5) was attempted The rate of the phosphatase reaction was, how-

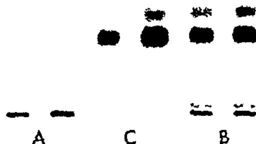


Fig 4 Chromatographic Demonstration of Phospholipase C Activity Crude filtrate and lecithin were incubated as described in the text

Fig 4a Samples were extracted with chloroform methanol (2 1 v/v) and concentrated to 1/6 of their volume Portions of 20 and 40 µl were applied to silica gel plates and developed in light petroleum ethyl acetate (75 25 v/v) A Before incubation B after 5 hrs incubation C L 1 2 diglyceride diluted 1 10 (v/v) in chloroform methanol (2 1 v/v) Lecithin remained at the point of application

ever, repeatedly found to be lower than the reaction rate of phospholipase C

DISCUSSION

In preceding studies on the *A calcoaceticus* haemolysin a broth culture filtrate was employed The high exogenous protein content of this medium imposed difficulties on the separation and characterization of the



Fig 4b Trichloracetic acid filtrate (60 µl) A before incubation B after 5 hrs incubation and 20 µl 25 mM phosphorylcholine (C) were applied to the paper and developed in butanol acetic acid water (60 15 25 v/v/v)

haemolysin The cultivation technique described gave a satisfactory yield of haemolysin under conditions suitable for further studies of the enzyme

Asparagine was found to be essential for haemolysin production, but the mechanism involved has not been investigated

A molecular weight of 25–35 000 is consistent with the results of previous gel filtration experiments (7) and with the finding that the haemolytic activity was retained by a Diaflo Ultrafiltration membrane UM 20E (molecular weight cut off 15–25 000) The molecular weight lies in the same range as that of phospholipase C (a toxin) of *Clostridium perfringens* (31 000) (2)

The isoelectric point of the main haemolytic fraction (pH 6 75) agrees with the slow electrophoretic mobility on cellulose acetate at pH 8 6 (7), and with the disc electrophoresis pattern (unpublished data) Coincidence of the peaks of haemolysis and acid soluble phosphorus gives further evidence of the identity of the haemolytic and phospholipase C activities A small fraction at pH 4 5–4 75 was also found in a preliminary isofocusing experiment employing broth culture filtrate but has not yet been further examined Among other possibilities it may represent a distinct molecular species or a fraction of the same enzyme associated with some other cell component

Phospholipase C activity was documented by chromatographic detection of the split products of purified lecithin 1 2 diglyceride and phosphorylcholine On TLC the patterns of standard and sample diglycerides were identical each appearing as two distinct spots The reason for this is not clear

Further investigations of the properties of phospholipase C of *A calcoaceticus* should preferably be performed on a purified enzyme preparation

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REACTION OF STAPHYLOCOCCAL PROTEIN A WITH RABBIT IMMUNOGLOBULINS

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Results are presented to show that protein A from *Staphylococcus aureus* strain Cowan I elicits the synthesis of rabbit IgG which reacts with protein A through the Fab region IgM from an anti protein A serum obtained 9 days after first injection of antigen appeared to react with crude protein A It was also found that protein A and protein B are antigenically similar In addition protein A contains distinctive determinants which appear to be responsible for the binding of protein A to the Fc region of IgG

Crude protein A from *Staphylococcus aureus* strain Cowan I is known to contain several serologically active components (20 5–9) Amongst these are two precipitinogens denoted protein A and protein B (7) Whereas protein B precipitates only with immune sera (7 10) protein A also reacts with normal human serum and the serum of a number of animal species (1 2 10 14) This latter reactivity has been attributed to the apparently non specific binding of protein A to the Fc regions of IgG molecules (1 2 11 10 13 15)

Although it is generally agreed that normal rabbit serum fails to precipitate with crude protein A direct precipitation of IgG isolated from normal rabbit serum has been reported (3) No such reactivity has been observed at this laboratory (10) and similar results have been reported from elsewhere (16 12)

Precipitins reacting with crude protein A are readily detected in the serum of rabbits immunized with either formalin killed *Staph aureus* strain Cowan I (20 6 7) or preparations of crude protein A (16) It is clear from the studies of *Forsgren* & *Sjöquist* (3) and the later studies of *Grov et al* (10) that protein A reacts with the Fc regions of IgG in immune rabbit sera However neither of these studies rule out the possibility of Fab reactivity for protein A

Recently *Forsgren* & *Sjoquist* (4) and *Kronvall* (12) presented evidence which was claimed to demonstrate reactivity for protein A associated with the F(ab) fragments of IgG isolated from rabbit antisera to crude protein A As crude protein A was used as both immunizing and test antigen in these studies it is possible that the F(ab) reactivity was confined to anti protein B antibodies (cf *Grov et al* (10)) In view of this possibility it was considered worthwhile to examine the reactivity of antisera from rabbits immunized with purified protein A In addition the reactivity of anti protein B sera has been examined

Received 15 v 71

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MATERIALS AND METHODS

Antigens Crude protein A and the purified antigens protein A and protein B were prepared from *Staph aureus* strain Cowan I as previously described (7)

Experimental animals New Zealand white rabbits bred and housed under conventional laboratory conditions at the animal house of this institute were used for the experiments

Sera and antisera Human serum obtained from healthy blood donors was pooled before use Antisera to *Staph aureus* strain Cowan I (Cowan I sera) were prepared in rabbits as described previously (10) Anti protein A and anti protein B sera were raised in rabbits given intra muscular injections of a saline suspension of the respective antigen emulsified in an equal volume of Freund's complete adjuvant (Difco) Two injections of antigen (0 5 mg/dose) were given 3 weeks apart and blood samples were collected before and at intervals after each injection A goat antiserum to rabbit serum was obtained from Miss Bodil Larsen The Gade Institute Department of Microbiology

Isolation of IgG IgG was precipitated from serum samples using 1 33M $(NH_4)_2SO_4$ and after reprecipitation twice with the same solution it was desalted on G 25 Sephadex (Pharmacia) before being lyophilized The different preparations were shown to be free of other serum proteins by immuno-electrophoretic analyses using the goat anti serum to rabbit serum

Enzymic digestions of IgG Isolated IgG was digested with papain and pepsin (Sigma) according to the procedures described by *Forsgren* (1) and *Grov et al* (10) respectively

Fractionation of serum Samples of serum were fractionated on diethyl amino ethyl (DEAE) Sephadex and G 200 Sephadex (Pharmacia) as described by *Macken ie* (17) and *Outteridge et al* (21) The optical density (O D) of consecutive volumes of column effluent was determined at 280 mμ then pooled effluent volumes were concentrated using polyethylene glycol

Serological reactions Double diffusion in agar and ring test examinations were carried out as described previously (20 10) using antigen concentrations of 1 0 mg/ml Immunoelectrophoretic analyses were performed using an LKB apparatus as described by the manufacturer (LKB Produkter) Inhibition tests with isolated Fab and Fc fragments were performed by incubating crude protein A (0 4 mg/ml) with an equal volume of the preparation to be tested (5 0 mg/ml) at 37° C for 1 hr then overnight at 4° C The reactivity of the crude protein A was then tested in double diffusion in agar against normal human serum Absorptions of anti protein A and anti protein B sera were carried out by adding 0 5 mg of lyophilized antigen preparation to 0 25 ml of serum sample, incubating at 37° C for 2 hrs then overnight at 4° C sedimenting any precipitate by centrifugation and finally testing the absorbed serum in agar diffusion The absorptions were continued until antigen excess was detected

RESULTS

Reactivity of the antigen preparations In double diffusion in agar, crude protein A reacted with normal human serum and Cowan I serum as previously reported from this laboratory (20, 5, 7) When these sera were placed in adjacent wells a distinct spur was sometimes seen in the common precipitin (protein A) line In every instance in which this phenomenon was observed, the precipitin line of Cowan I serum spurred over that of normal human serum The purified antigen preparations reacted in agar diffusion as was previously reported (7) Thus protein A reacted with both Cowan I serum and normal human serum to produce a single precipitin line in each instance and protein B reacted only with Cowan I serum producing a single precipitin line

Response to immunization Prior to immunization the sera of all rabbits failed to precipitate with crude protein A in agar diffusion and in ring test Anti protein A sera precipitated in agar diffusion with crude protein A at titres of 64–128 by 3 weeks after first injection of antigen and by 2 weeks after the second injection titres of 128–256 were recorded At these times the precipitin titres of anti protein B sera for crude protein A were 32 64 and 64–128 respectively

Reactivity of antisera Both anti protein A and anti protein B reacted with crude protein A to produce two distinct precipitin lines in agar diffusion When the antisera were reacted with the purified antigen preparations complete cross reactivity was observed Thus anti protein A reacted with protein B to produce a single precipitin line and similarly a single line was obtained when anti protein B was reacted with protein A (Figs 1 and 2)

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protein A was reacted with anti protein A *anti protein B and Cowan I serum were confluent* (Fig 1) but all three lines spurred over that between protein A and normal human serum (Fig 2) When protein B was reacted with anti protein A anti protein B and Cowan I serum placed in adjacent wells the resultant precipitin lines were confluent (Fig 1)

Absorption of anti protein A and anti protein B with crude protein A completely exhausted the respective sera of precipitins for protein A protein B and crude protein A All precipitating activity of anti protein B was removed by absorption with protein A (Fig 3) In contrast absorption of anti-protein A *with protein B failed to* eliminate all the precipitating activity The resultant sera still reacted with protein A to give a precipitin line which was confluent with the precipitin line of normal human serum and showed a reaction of partial identity with the line of Cowan I serum (Fig 3)

Reactivity of IgG and F(ab)₂ Fab and Fc fragments Whereas IgG (2 mg/ml) isolated from normal rabbit serum failed to react in agar diffusion with crude protein A IgG from anti protein A serum at the same concentration reacted strongly The pepsin digest of this latter preparation reacted with crude protein A to produce a weak precipitin line *in agar diffusion and although the Fab pre*paration failed to precipitate directly in agar diffusion it was found to inhibit the reaction of crude protein A with normal human serum Similar but apparently weaker inhibitory activity was obtained when the Fc preparation of IgG isolated from anti protein A serum was tested (Fig 4)

Fractionation of anti protein A serum Three OD peaks were detected after fractionation of serum on G 200 Sephadex Immunoelectrophoretic analyses revealed that IgM was associated with the first and IgG with the second OD peak When a serum obtained 9 days after first injection of antigen was fractionated on G 200 Sephadex precipitating activity for crude protein A was detected in the fractions of the first and second OD peaks (Fig 5a) Fractionation of *a serum obtained 3 weeks after first injection* showed that precipitating activity was associated primarily with the fractions of the second OD peak (Fig 5b)

When a serum obtained 3 weeks after first injection was fractionated on DEAE Sephadex 5 major OD peaks were obtained Precipitins for crude protein A were confined solely to the first 3 peaks Immunoelectrophoretic analyses showed that the first OD peak contained slow migrating IgG (IgG_2) and fast IgG (IgG_1) was associated with the fractions of the second and third OD peaks (Fig 5c)

DISCUSSION

It seems reasonable to conclude that protein A elicits the synthesis of immunoglobulins capable of reacting with this antigen through the Fab-region of the antibody molecules This is most evident from the results obtained with the products of enzymic digestion of IgG from anti protein A sera Although it is conceded that contamination of these preparations with undigested IgG can not be overlooked the additional evidence for Fab reactivity is strong

The finding that the precipitin lines of Cowan I anti protein A and anti protein B *sera spurred over the line of normal human* serum in agar diffusion against protein A (cf Fig 2) is compatible with antibodies to protein A having Fab-reactivity It is well documented that IgG in normal human serum reacts with protein A due to binding of protein A to the Fc regions (2 11 13 15) and it has recently been shown that antibodies to protein B react with protein B through the Fab regions of the immunoglobulin molecules (10) It is suggested that anti protein B antibodies react with both protein A and protein B through the Fab regions and that similar reactivity for protein B is displayed by antibodies to protein A Moreover antibodies to protein A react with protein A through Fab and/or Fc region and absorption of anti protein A with protein B

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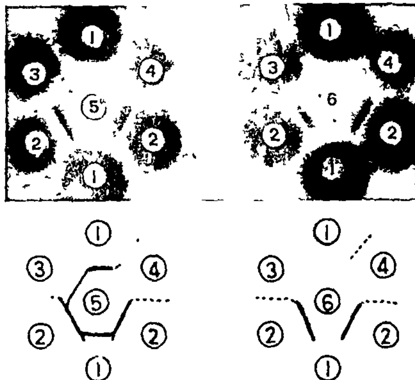


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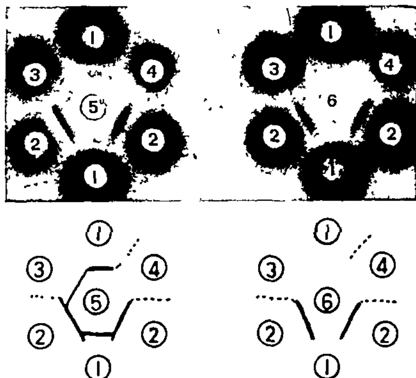


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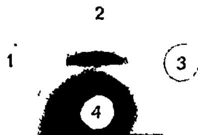


Fig 4 Double diffusion in agar of (1) crude protein A previously incubated with Fc fragments of IgG from anti protein A serum (2) crude protein A (0 2 mg/ml) and (3) crude protein A previously incubated with Fab fragments of IgG from anti protein A serum The precipitin pattern was developed using normal human serum (4)

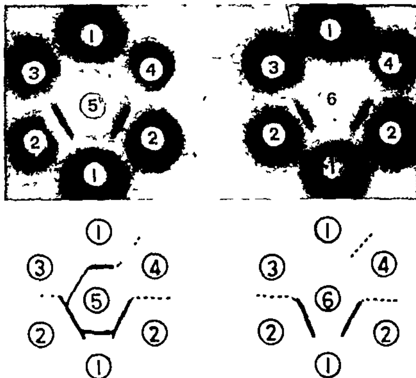


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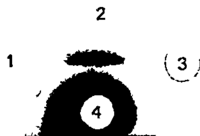


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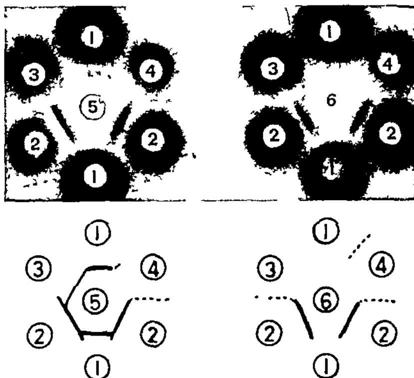


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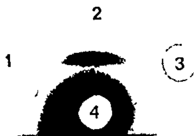


Fig 4 Double diffusion in agar of (1) crude protein A previously incubated with Fc fragments of IgG from anti protein A serum (2) crude protein A (0 2 mg/ml) and (3) crude protein A previously incubated with Fab fragments of IgG from anti protein A serum The precipitin pattern was developed using normal human serum (4)

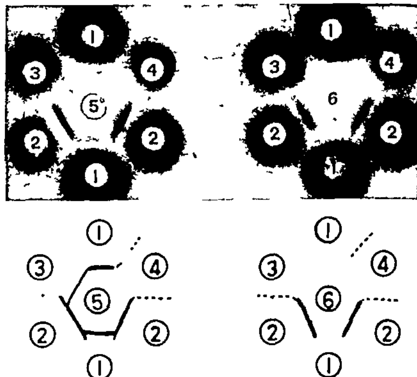


Fig 3 Double diffusion in agar of (1) normal human serum (2) Cowan I serum (3) anti protein A absorbed with protein B and (4) anti protein B absorbed with protein A The precipitin arcs were developed with (5) protein A and (6) protein B Note that the precipitin line of Cowan I serum spurred over the line of absorbed anti protein A and that this latter line was confluent with the line of normal human serum Also note that the absorbed sera contained excess absorbing antigen

Results obtained by *Forsgren & Sjöquist* (4) were interpreted as evidence for protein A reactivity associated with F(ab) fragments of IgG isolated from antisera to crude protein A This result would appear to be equivocal as it is possible that anti protein B activity was detected More recently Kronvall (12) obtained results which showed reactivity for crude protein A associated with $F(ab)_2$ fragments of IgG isolated from rabbit antisera to crude protein A Although this latter worker used crude protein A as immunizing and test antigen the system chosen to test for Fab reactivity possibly demonstrated anti protein A activity The failure of other workers to demonstrate Fab reactivity for protein A (3 10 emphasizes the need to have potent antisera

In view of the results obtained in the present experiments for rabbit immunoglob

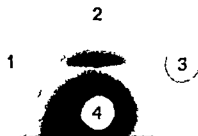


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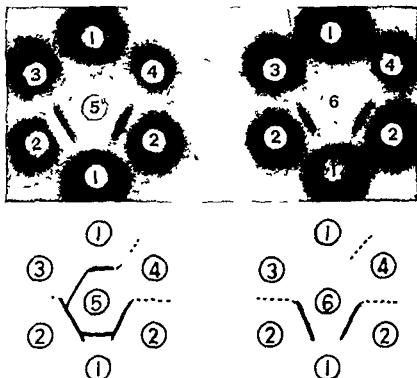


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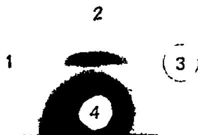


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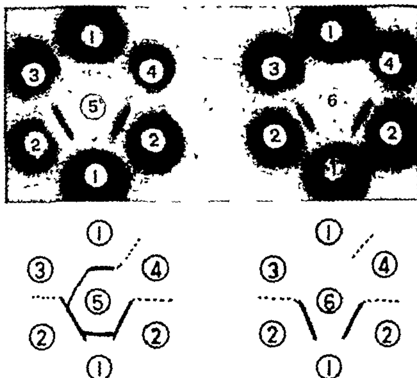


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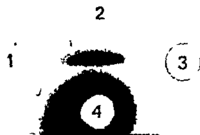


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Acta path microbiol Scand Section B 79, 801-804, 1971

IgA AND IgM REACTING WITH STAPHYLOCOCCAL PROTEIN A

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IgM in guinea pig antisera obtained 10 days after immunization with purified protein A, and IgA in human colostrum from apparently healthy mothers, reacted with protein A These findings are consistent with antibodies to protein A having Fab- as well as Fc reactive sites

Protein A from *Staphylococcus aureus* is known to react with normal IgG from humans and a number of animal species (6, 4, 5, 8, 12, 13, 11) Until recently this reaction was thought to be due solely to binding of protein A to the Fc regions of the IgG molecules but recent experiments from this laboratory (14) demonstrated Fab reactivity associated with IgG in rabbit antisera to protein A During the course of these investigations evidence was obtained which suggested that IgM in anti-protein A sera reacted with protein A It therefore seemed worthwhile to examine the reactivity of IgM from guinea-pig antisera for reactivity with this antigen In addition the reactivity of immunoglobulins in human colostrum with protein A has been examined

MATERIALS AND METHODS

Antigens Crude protein A and purified protein A were isolated from *Staph aureus* strain Cowan I according to the procedures described previously (7)

Experimental procedure Three adult male guinea pigs bred and housed under conventional laboratory conditions at the animal house of this Institute were used for the experiments Each animal was injected intra muscularly with a saline suspension of purified protein A (0 5 mg) emulsified in an equal volume of Complete Freund s adjuvant Blood samples were obtained immediately before immunization and when the animals were killed by exsanguination 10 days after immunization

Two samples of human colostrum from apparently healthy mothers were obtained from the maternity department of Haukeland Hospital, Bergen Casein free milk whey was prepared by acidifying (to pH 4 6 with 1N HCl) skimmed milk which was subsequently centrifuged to sediment the precipitated casein The whey samples were then neutralized using 1N NaOH

Fractionation of serum and whey Serum and whey samples (4–5 ml) were fractionated on a column (2×90 cm) of G 200 Sephadex (Pharmacia) according to the procedure described previously (14) The optical density of each effluent fraction was recorded at 280 mμ, then pooled effluent volumes were concentrated using polyethylene glycol (Fluka)

Serological methods Ring test and double diffusion in agar were carried out as outlined previously (8) Immunoelectrophoretic analyses of serum and whey and fractions of serum and whey were performed using an LKB apparatus according to the manufacturer s recommendations (LKB Produkter) The immunoelectrophoretic patterns were developed using antisera obtained from Hyland Laboratories

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Human colostrum was absorbed with antisera specific for human IgG (anti-γ) and IgM (anti-μ) which were obtained from Hyland Laboratories The absorptions were carried out by adding 0 1 ml of anti-γ and 0 1 ml of anti μ to 0 2 ml of colostral whey, incubating at 37° C for 2 hr then overnight at 4° C before centrifuging to remove precipitated material Absorptions were continued until IgG and IgM were removed as shown by agar diffusion against anti γ and anti-μ Finally, the reactivity with protein A of the absorbed whey was examined in ring test The antisera used for absorption (anti-γ and anti μ) did not react with protein A in ring test

RESULTS

Reactivity of serum and whey with protein A

The pre-immune sera from the guinea-pigs precipitated with crude protein A in agar diffusion at titres of 4–8 and reacted strongly in ring test with crude protein A When the sera obtained *10* days after immunization were examined in agar diffusion, titres of 32–64 were recorded Both samples of human colostrum reacted with crude protein A in agar diffusion and titres of 16–32 were detected in ring test

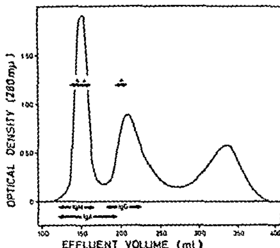


Fig 2 Optical density profile for G-200 Sephadex fractionation of 5 ml of human colostral whey. The immunoglobulins detected in effluent fractions by immunoelectrophoretic and double diffusion in agar analyses are shown below the profile Fractions found to react with crude protein A in ring test are also indicated
+ + strong reaction, + weak reaction

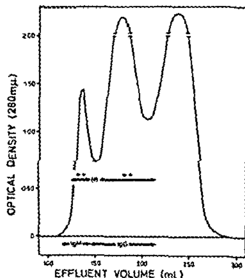


Fig 1 Optical density profile for G 200 Sephadex fractionation of 4 ml of guinea-pig serum obtained 10 days after intramuscular injection of purified protein A incorporated in Freund's complete adjuvant The immunoglobulins detected in effluent fractions by immunoelectrophoretic analyses using anti-guinea-pig serum and anti-guinea-pig immunoglobulin serum are shown below the profile Concentrated, pooled effluent fractions found to react with crude protein A in ring test are also indicated + + strong reaction, (+) weak reaction

Fractionation of serum and whey

Guinea-pig serum When immune guinea-pig serum was fractionated on G-200 Sephadex, a typical optical density profile with 3 peaks was obtained (Fig 1) The fractions associated with the first peak were found to contain IgM as the only detectable immunoglobulin component (using antiserum to guinea-pig serum and antiserum to guinea-pig immunoglobulins) and these fractions reacted strongly with protein A in ring test Only weak protein A reactivity was detected in the fraction associated with the trough region between the first and second optical density peaks and this fraction contained trace amounts of IgG The fractions of the second optical density peak reacted strongly with protein A These fractions were found to

contain IgG as the only immunoglobulin component

Human colostral whey The optical density profile obtained when whey was fractionated is shown in Fig 2 Protein A reactivity was detected in the fractions associated with the first peak and weak reactivity was associated with one fraction in the second peak Immunoelectrophoretic and double diffusion in agar analyses using antisera to human serum and antisera specific for human IgA, IgG and IgM revealed that IgM and IgA were associated with the fractions of the first peak and IgG was detected in the fractions of the second peak.

Reactivity of absorbed whey After absorption with anti γ and anti μ the human colostrum samples still reacted strongly with protein A in ring test

DISCUSSION

The present experiments clearly show that immunoglobulins other than IgG react with protein A Recently *Forsgren* (4) showed that IgG_1 and IgG_2 but not IgM, in the sera of guinea pigs immunized with egg albumin reacted with protein A due to binding of this antigen to the Fc region of these immunoglobulins (5) It has also been shown that human myeloma IgA and IgM globulins do not react with protein A (16) In view of these findings it appears that guinea pig IgM and human IgA and IgM lack the Fc-reactive structures characteristic of the IgG molecules of many species (6 16 5 8 11) Moreover it appears that the reaction of protein A with IgM in early immune guinea pig sera and rabbit immune sera (14) and IgA in human colostrum is due to binding of protein A to the Fab-regions of these immunoglobulins Final proof of this must however await the results of further investigations

That IgA present in human colostrum reacted with protein A is of particular interest It is well documented that IgA (most of which is present as a dimer) is the predominant immunoglobulin in human colostrum (3, 17, 2) Although the possibility can not be discounted that the mothers from whom the colostrum was obtained had been locally stimulated by staphylococcal antigens, it is considered likely that the secretory immunoglobulin system of the mammary gland was synthesizing IgA with specificity for protein A in the absence of local antigenic stimulation In this connection it has been reported that antibody with anti bacterial and anti viral specificity appears in the colostrum of mothers not thought to have been locally stimulated (10, 1, 2, 15), and it was recently demonstrated that specifically stimulated lymphoid cells, originating from systemic lymph nodes, colonize extra vascular tissue sites and probably provide for a source of local antibody (9)

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Acta path microbiol Scand Section B 79, 805-811, 1971

LOCAL IMMUNIZATION OF GUINEA PIG MAMMARY GLAND WITH STAPHYLOCOCCAL ANTIGENS

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Immunization of the mammary glands of pregnant guinea pigs with *Staphylococcus aureus* or isolated mucopeptide induced a local immune response that persisted throughout lactation Precipitin arcs corresponding to IgG, IgM, albumin and a protein of β electrophoretic mobility were seen in immunoelectrophoretic plates of whey developed using antiserum to guinea pig serum When antiserum to guinea pig immunoglobulins was used a strong arc in the β position and a weak IgG arc appeared The protein of β electrophoretic mobility was the only component in whey from immunized glands, which reacted with polysaccharide A (teichoic acid) in immunoelectrophoresis No reaction with this antigen was observed for whey from non immunized glands or serum It is suggested that the protein of β electrophoretic mobility detected in guinea pig whey is analogous to secretory IgA from humans and other species

Staphylococcal infections of the mammary glands of humans and domestic animals are often difficult to treat with therapeutic agents because of the occurrence of many strains of *Staph aureus* that are resistant to such agents Thus it is possible that specific stimulation of the local immune response would be of benefit in preventing staphylococcal infections of the mammary gland

This approach has been adopted by *Lascelles* and co workers (17, 19, 14) who have demonstrated that local immunization of pregnant sheep shortly before parturition with staphylococcal vaccines is effective in protecting immunized mammary glands from experimental mastitis induced during lactation Similar results have also been reported by *Derbyshire* & *Smith* (2) who used goats as experimental animals

Although it is clear that local immunization with staphylococcal vaccines is efficacious in protecting sheep and goats from experimental mastitis, the importance of the different antigens of staphylococci in eliciting this protective immunity has not been resolved Accordingly, in the present experiments, attempts have been made to determine which of the major cell wall antigens of *Staph aureus* are capable of eliciting an immune response in mammary gland tissue Guinea pigs have been chosen as experimental animals as these were the only animals readily available for the studies

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MATERIALS AND METHODS

Experimental animals Pregnant guinea pigs bred and housed under conventional conditions at the

animal house of this Institute were used Only those animals found to be free of mammary gland abnormality were chosen The guinea pigs were of variable but unknown parity

Antigens Formalin killed *Staph aureus* strain Cowan I were prepared as described earlier (16) Mucopeptide and crude protein A were obtained from *Staph aureus* strain Cowan I according to the pocedures described by *Park & Hancock* (20) and *Grov et al* (6), respectively Polysaccharide A (poly A) from *Staph aureus* strain Wood 46 was prepared as outlined by *Grov* (5)

TABLE 1 *Experimental Plan*

Antigen preparation	No of animals	Left gland
Formalin killed *Staph aureus* strain Cowan I 5×10^6 organisms/ml	5	0 2 ml
Crude protein A 5 mg/ml	5	0 2 ml
Mucopeptide 5 mg/ml	5	0 2 ml

Experimental procedure Antigen preparations were injected into 4 subcutaneous sites in the region of the left mammary gland of each guinea pig according to the plan outlined in Table 1 The preparations were dissolved or suspended in sterile phosphate buffered saline (p b s) It was apparent from the results of preliminary experiments (*Mc Dowell & Grov* unpublished data) that an optimal local response occured if antigen was administered 1 2 weeks before paturition In view of this finding injections were given approximately 1 week before the estimated date of parturition

Blood samples were collected before immunization and at intervals thereafter and milk samples were obtained on the day of parturition and at periods during lactation Casein free milk whey was obtained by acidification of skimmed milk to pH 4 6 with 1N HCl and subsequent centrifugation to sediment the precipitated casein

Serological methods Staphylococcal agglutination assays were performed in test tubes ($0\,5 \times 5$ cm) using formalin killed *Staph aureus* strain Cowan I organisms that had been harvested from nutrient agar The organisms were suspended in p b s containing sodium azide (1 2 500) to give a suspension of 0 5 per cent (v/v) Mucopeptide agglutination tests were also performed in test tubes ($0\,5 \times 5$ cm) using a suspension of mucopeptide (0 5 mg/ml) in p b s, containing sodium azide

Double diffusion in agar tests with crude protein A (1 0 mg/ml) were carried out as described previously (6) Immunoelectrophoretic analyses were performed using an L K B apparatus according to the recommendations of the manufacturer (L K B Produkter) The immunoelectrophoretic patterns were developed with rabbit antiserum to guinea pig serum rabbit antiserum to guinea pig immunoglobulins (both obtained from Hyland Laboratories) or a solution of poly A (2 0 mg/ml) in p b s

Adsorption of whey antibody to mucopeptide Samples of whey obtained on the day of parturition from the immunized glands of the guinea pigs injected with mucopeptide were pooled and similarly whey samples from the non immunized glands of these animals were pooled A total of 10 mg of mucopeptide were added to 1 ml of each pooled sample and the suspensions were incubated at 37° C for 1 hr then overnight at 4° C After thorough washing (x 6) by centrifugation and re suspension in p b s, 4 drops of HCl/saline (pH 3 0) were added to each preparation of sensitized mucopeptide Finally the mucopeptide was sedimented by centrifugation and the supernatants were tested in immunoelectrophoresis using the antiserum to guinea pig immunoglobulins and the antiserum to guinea pig serum to develop the patterns

RESULTS

Response to Local Immunization

The inflammatory responses that followed antigen injections were very mild and no detectable abnormalities were seen 2 3 days after injection At parturition and during lactation milk production from immunized and non immunized glands was similar

Antibody Response to Immunization with Whole Bacteria

The sera of all animals contained high levels of staphylococcal agglutinins prior to immunization and agglutination titres in serum were usually increased twofold by immunization (Table 2) In whey samples obtained on the day of parturition agglutination titres were usually lower than in corresponding serum samples A distinct difference in the levels of staphylococcal agglutinins in whey from immunized and non immunized glands was found for 2 animals (nos 27 and 33) on the day of parturition but no differ-

TABLE 2 *Staphylococcal Agglutination Titres in Samples of Serum and Whey from Guinea Pigs Immunized with Staphylococci*

Guinea pig number		Days between injection and parturition	Sample obtained a	b	c	d
	S		4096	8192	8192	—
27	L	15	—	1024	512	256
	R		—	256	128	64
	S		4096	4096	4096	—
32	L	6	—	128	256	64
	R		—	128	64	16
	S		2048	4096	4096	—
33	L	6	—	4096	128	128
	R		—	128	64	64
	S		2048	2048	4096	—
35	L	6	—	512	64	256
	R		—	512	16	64
	S		4096	8192	8192	—
40	L	12	—	1024	64	—
	R		—	1024	16	—

Serum (S) and whey from immunized (L) and non immunized (R) glands were obtained immediately before immunization (a), on the day of parturition (b) 1 week (c) and 2 weeks (d) after parturition Not tested ( )

ence was observed for the other 3 animals However, clear differences in the levels of agglutinins were detected in whey samples obtained 1 and 2 weeks after parturition The titres in whey from immunized glands were always higher than in corresponding samples from non immunized glands at these times

When whey samples obtained on the day of parturition were tested in agar diffusion against crude protein A precipitins were detected in the samples from the immunized glands of 2 animals (nos 27 and 33) No precipitins were detected in whey from the immunized glands of the other animals and similarly whey from the non immunized glands of all animals failed to precipitate with crude protein A The whey samples from 1 animal (no 27) were tested throughout lactation and precipitins for crude protein A were detected in whey from the immunized, but not the non immunized, gland for 3 weeks after paturition

Response to Immunization with Crude Protein A

Prior to immunization precipitins for crude protein A were detected in the sera of all guinea pigs In most instances titres were not appreciably increased by immunization and at parturition no precipitins were detected in whey samples

Response to Immunization with Mucopeptide

The sera from all guinea pigs agglutinated mucopeptide prior to immunization (Table 3) On the day of parturition serum titres were 2–16 times higher than before immunization Agglutination titres were always higher in whey from immunized than non immunized glands on the day of parturition and this difference was evident in samples obtained 1 and 2 weeks after parturition

TABLE 3 *Mucopeptide Agglutination Titres in Samples of Serum and Whey from Guinea Pigs Immunized with Mucopeptide*

Guinea pig number		Days between injection and parturition	Sample obtained a	b	c	d
50	S	11	128	1024	512	1024
	L		—	1024	256	256
	R		—	256	64	64
54	S	4	256	512	2048	—
	L		—	1024	512	—
	R		—	256	128	—
57	S	4	256	1024	512	512
	L		—	1024	256	512
	R		—	256	64	128
58	S	10	128	2048	1024	512
	L		—	1024	512	1024
	R		—	256	64	64
59	S	14	256	4096	1024	1024
	L		—	1024	256	512
	R		—	128	—	32

Serum (S) and whey from immunized (L) and non immunized (R) glands were obtained immediately before immunization (a), on the day of parturition (b) 1 week (c) and 2 weeks (d) after parturition Not tested ( )

Immunoelectrophoretic Analyses of Whey Samples

When the rabbit antiserum to guinea pig serum was used to develop immunoelectrophoretograms of whey obtained on the day of parturition 4 clear precipitin arcs appeared (Fig 1) These corresponded with the precipitin lines of IgG IgM albumin and a protein of β-electrophoretic mobility The latter component was not readily detected in serum patterns (Fig 1) In the patterns of whey developed using the antiserum to guinea pig immunoglobulins a strong arc in the β position was observed and this apparently fused with a weaker arc of slower electrophoretic mobility (Fig 2) Only one precipitin line corresponding to IgG was detected in the serum patterns (Fig 2)

The protein of β-electrophoretic mobility was the only component in whey from glands immunized with whole bacteria (obtained on the day of parturition) that reacted with poly A No reaction was observed when serum samples whey from non immunized glands and whey from both non immunized and immunized glands collected during lactation were examined

Immunoelectrophoretic Analyses of the Eluates Obtained from Mucopeptide Sensitized by Whey Samples

The eluates from mucopeptide sensitized by whey from both immunized and non immunized glands were found to contain only the protein of β electrophoretic mobility when the precipitin patterns were developed with the antiserum to guinea pig immunoglobulins No precipitin arcs were detected when anti guinea pig serum was used

DISCUSSION

It has recently been reported that local immunization of the mammary glands of pregnant sheep (12 18 13) and rabbits (4 3) elicits a local production of specific anti

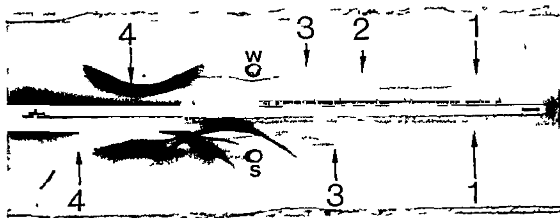


Fig 1. Immunoelectrophoretic plate of guinea pig whey obtained on the day of parturition from a gland immunized with killed *Staph aureus* (w) and guinea pig serum (s) Patterns were developed using anti serum to guinea pig serum 1 = IgG, 2 = protein of β electrophoretic mobility, 3 = IgM*, 4 = albumin * Not distinct in the photograph

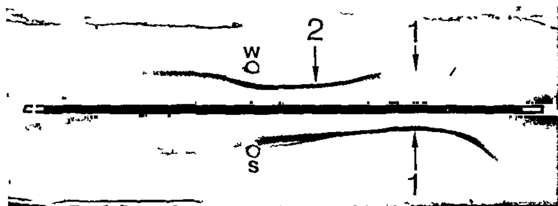


Fig 2 Immunoelectrophoretic plate of guinea pig whey obtained on the day of parturition from a gland immunized with killed *Staph aureus* (w) and guinea pig serum (s) Patterns were developed using antiserum to guinea pig immunoglobulins 1 = IgG 2 = immunoglobulin with β-electrophoretic mobility

body The present findings are consistent with these earlier reports and demonstrate that immunization of the mammary glands of pregnant guinea pigs is effective in stimulating a local immune response

Relatively high levels of specific antibody were present in the secretions of the non immunized glands and it is possible that this antibody was partly of serum origin It is also conceivable that previous local antigenic stimulation with staphylococcal antigens had occurred as the previous history of the animals was unknown Indeed, staphylococci are common amongst the microbial flora of guinea pigs However, it is recalled that antibody with anti viral and anti bacterial specificities has been found in the colostrum of humans not thought to have been locally stimulated (10, 11, 1 15, 9) In connection with the latter, it was recently hypothesized (7) and later confirmed (8) that specifically-stimulated lymphoid cells originating from lymph nodes extravasate into tissues and probably provide for a source of local antibody

Clearly, the major structural components

of the staphylococcal cell wall (*viz* mucopeptide and teichoic acid) and crude protein A elicited the production of specific antibody by guinea pig mammary gland Further work needs to be done to define which, if any, of these antigens are important in eliciting the synthesis of antibodies capable of conferring protection against local staphylococcal infections Recent results published by *Shayegani et al* (22) indicate that human serum contains factors which enhance the phagocytosis and killing of *Staph aureus* and that these factors are removed by absorption with mucopeptide but not by absorption with teichoic acid or crude protein A However, the importance of antibodies in serum acting as opsonins would appear to be equivocal in view of results presented by *Shayegani* (21)

The finding that crude protein A, when injected alone, failed to stimulate a detectable local immune response is perhaps surprising in view of the results obtained using whey from glands immunized with whole bacteria However, crude protein A is poorly immunogenic when administered intra muscularly to guinea pigs in the absence of adjuvant (*McDowell & Grov*, unpublished data) That crude protein A failed to stimulate a detectable local immune response in guinea pig mammary gland is consistent with the finding that monomeric flagellin from *Salmonella adelaide* is much less immunogenic than polymeric flagellin in the mammary gland of the sheep (13)

The protein of β electrophoretic mobility detected in whey reacted with an antiserum specific for guinea pig immunoglobulins On the basis of this result it is clear that this component is an immunoglobulin and it was evident that it was the major immunoglobulin component of whey It seems reasonable to conclude that this immunoglobulin is analogous to secretory IgA found in the colostrum and milk and other external secretions of humans and many mammalian species (23, 24)

Based on the results obtained when immunoelectrophoretic patterns of whey were developed with poly A it is suggested that local immunization with whole bacteria elicited a local synthesis of IgA specific for this antigen It is considered that the other antigens also elicited the synthesis of IgA although none of the results can substantiate this From the immunoelectrophoretic results obtained for the eluates from sensitized mucopeptide preparations it is clear that IgA with specificity for mucopeptide is present in milk from non immunized glands Thus, it appears that the secretory immunoglobulin system in guinea pig mammary gland is synthesizing specific antibody probably in the abscence of specific local antigenic stimulation (cf 7, 8, 15)

G McDowell wishes to thank the *Australian Dairy Produce Board* for an Overseas Post Graduate Studentship

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Acta path microbiol Scand Section B 79, 812-818, 1971

IMMUNOCHEMICAL CHARACTERIZATION OF *STAPYLOCOCCUS EPIDERMIDIS* AND *MICROCOCCUS* CELL WALLS

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Undigested cell walls from two *Staphylococcus epidermidis* and two *Micrococcus* strains have been isolated and subjected to chemical and serological examinations Glycerol, the only sugar alcohol observed, and hexoses were present in all wall preparations Cell walls from one of the micrococcal strains contained large amounts of alanine and only trace amounts of glycine Serine was present in higher amounts in *Staph epidermidis* than in *Micrococcus* walls The precipitinogens found in extracts of whole bacteria were also present in the cell walls but apparently behaved as haptens One precipitinogen present in the wall of a *Staph epidermidis* strain was very difficult to extract It resisted periodate oxidation but behaved as a teichoic acid in immunoelectrophoresis The staphylococcal cell walls sensitized both normal and tanned sheep erythrocytes for agglutination in antisera whereas the micrococcal cell walls did not The agglutinogens of whole bacteria were also present in the wall preparations although in reduced amounts

Serological studies of undigested cell wall preparations from *Staph aureus* (34, 23, 17) have demonstrated the presence of the same precipitinogens, agglutinogens, and antigens that sensitize red cells found in whole bacteria The chemical basis of some of these antigenic activities has been shown to be due to well characterized components of the cell wall Teichoic acids (3) give characteristic precipitin lines with antisera by double diffusion in agar (19, 9, 31) The antigenic specificities displayed are dependent on the nature of the polyol, the sugar, and the configuration of the sugar The wall teichoic acid of *Staph aureus* is of the ribitol type with α- or β-linked N acetylglucosaminyl residues (9) In *Staph epidermidis* cell walls the teichoic acid is a polymer of α- or β-glucosyl glycerol phosphate (7, 8 9, 31) The *Micrococcus* group seems to comprise strains which have either ribitol (containing glucosaminyl residues) glycerol (with various sugar residues) or no teichoic acid in their cell walls (4) Various polysaccharides have been extracted from walls of the latter type

Also the mucopeptide structure of the cell wall is known to contain antigenic determinants (30 2, 24, 20, 12, 13), the specificities of which most probably depend on the characteristic linkages, the substitution of sugars, and the type of peptide cross bridging

Received 25 vi.71

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(16) *Micrococcus* strains generally differ from staphylococci in the peptide bridging (11)

The present paper deals with studies of undigested walls from two *Staph epidermidis* and two *Micrococcus* strains

MATERIALS AND METHODS

Strains Staph epidermidis strains 1254 (25) and 577 (1) and micrococcal strains *M luteus* 144 and *M conglomeratus* 84 (29) were selected for the preparation of cell walls In addition *Staph aureus* type strains Cowan I (30), 263 (21), and Wood 46 (18) were used for comparative serological examinations

Growth and harvesting The bacteria were grown on nutrient agar plates at 37° C for 18 hr then harvested by scraping and stored at –25° C until used

Preparation of cell walls Cell walls were pre pared according to the procedure of *Yoshida et el* (35) The cells were disintegrated in the X press at approximately –25° C Disintegrated cells were then washed several times with phosphate citrate buffer and M KCl, and submitted to density grad ent and pile centrifugations Dialysed and freeze dried cell wall preparations were checked for purity by chromatographic examination for nucleic acid components and by electron microscopy Negative staining was employed for the electron microscopic examinations, using a Siemens Elmi skop 1 m croscope Single drops of homogenized suspensions of cell walls in phosphotungstic acid

temperatures and for different time intervals (32) and also with formamide at 160° C for 30 min (10) Extracted walls were centrifuged dialysed and finally freeze dried

Bacterial extracts for sensitization of normal and tanned sheep erythrocytes (NSE and TSE) were prepared by suspending the bacteria in phosphate buffered saline, pH 7 2 (2 3 ml per g of wet bacteria), incubating for 18 hr at 37° C and then centrifuging at 10 000 g for 30 min To sensitize 10 ml of a 0 5 per cent suspension of NSE or TSE, 0 5 ml of the supernatant was used Heavy sus pens ons of bacteria were applied directly to the wells in agar gel precipitation tests

Chemical examinations Determinations of nitro gen and phosphorus were performed according to the methods referred to in (14) Portions of cell walls were hydrolysed with 0 1 N HCl for 2 hr at 100° C, or 3 N HCl for 3 hr at 100° C or with 6 N HCl for 18 hr at 105° C for chromatographic analyses The hydrolysates were evaporated to dryness *in vacuo*, dissolved in measured volumes of water and subjected to qualitative circular paper chromatography as described earlier (14) Quan titative determinations of amino acids in 6 N HCl hydrolysates were performed according to *Moore et al* (27) using an automatic amino acid analyzer (Beckman Spinco Model 120 B)

Periodate oxidation was performed in 0 1 M sodium periodate The cell wall preparations (1 mg per ml) were incubated at room temperature in the dark for 24 hr, then dialysed against distilled water

Serological examinations Rabbit antisera to whole cells (anti bacterial sera) were prepared as described by *Oeding* (28) Antisera to cell wall preparations were produced in rabbits given sub-cutaneous injections of cell walls emulsified in equal volumes of complete *Freund s* adjuvant The rabbits were given 3 injections (1 mg/dose) at intervals of 2 weeks and bled 2 weeks after the last injection

Bacterial slide agglutination tests were performed as described by *Oeding* (28), and *cell wall tube agglutination* as described in (17) *Double diffusion in agar, indirect haemagglutination* and *immunoelectrophoretic analyses* were carried out as in previous experiments (14, 30) Purified pre parations of polysaccharide A (poly A) (18), polysaccharide B (poly B) (26), polysaccharide AC (poly AC) (26), polysaccharide 263 (poly 263) (21), antigen D (22) protein A (14), *n* antigen (15), and I_3 teichoic acid (31) were included as references in the agar precipitation tests

RESULTS

All cell wall preparations were white in colour and no intact cells were observed in the electron microscopic examinations Neither were spots related to nucleic acids detected when chromatograms of hydroly-sates obtained with 0 1 N HCl were exposed to U V radiation

Marked variations in the chemical com position of the cell walls were observed (Table 1)

The amino acids usually found in muco-peptides (Lys, Glu A, Ala, Gly) were gener-ally present in the highest amounts in all preparations In this connection the cell walls of *M conglomeratus* 84 differed strikingly from the other preparations, having a very low content of glycine and a considerably higher content of alanine The *Staph epider-*

TABLE 1 *Quantitative Analyses of Cell Wall Preparations The Figures Represent μ mole/100 mg Cell Wall*

	Staph epidermidis		*Micrococcus*	
	577	1254	congl 84	luteus 144
Phosphorus	64 5	71 0	24 2	8 0
Nitrogen	542 8	692 8	542 8	657 1
Lysine	16 0	28 2	21 0	39 6
Aspartic acid	32 1	3 1	6 1	3 6
Serine	16.1	35 6	4 4	2 9
Glutamic acid } Muramic acid }	35 2	29 9	23 6	98 8
Glycine	28 7	110 6	6 8	46 7
Alanine	40 7	81 8	105 7	89 3
Arginine	8 6	0 3	3 7	0 8
Threonine	17 1	3 3	4 3	2 9
Proline	9 1	1 3	3 6	trace
Histidine	3 2	1 7	1 7	1 0
Valine	19 3	1 7	5 2	2 2
Isoleucine	15 7	1 7	3 6	12 2
Leucine	21 9	1 4	4 7	2 5

midis cell walls contained much more serine than the *Micrococcus* walls

The paper chromatographic analyses of sugars and sugar alcohols showed the presence of glycerol and hexoses in all cell walls (Table 2) Three unidentified sugar spots were observed on chromatograms of 3 N HCl hydrolysates from *M luteus* 144 walls After three runs in BuOH HAc H_2O (4 1 1), the R_f glucose values were for these sugars measured as 0 22 0 36 and 0 58

None of the cell wall antisera reacted with cell walls in double diffusion However precipitin lines were obtained when anti bacterial sera were used Both cell walls and extracts from *Staph epidermidis* 577 reacted with homologous anti bacterial serum to produce two strong and one weak precipitin lines One of the strong lines appeared close to the serum well after 4 6 hr at 37° C and was confluent with the C-line of poly AC This line also showed a reaction of partial identity with poly A when *Staph epidermidis* 577 and *Staph aureus* Wood 46 anti bacterial sera were used The second strong line appeared slowly and was situated close to the antigen well The antigens responsible for this and the weak precipitin line (appearing midway between serum and antigen wells) could not be identified with the reference antigens Extraction of cell walls with 5 per cent TCA at 100° C for 15 min removed the antigens responsible for the poly C and the weak precipitin lines but the other strong line could still be detected even after additional extraction with formamide for 30 min Further extraction of the walls with 10 per cent TCA at 45° C for 24 hr removed the residual precipitating activity This latter precipitinogen was not sensitive to periodate oxidation but behaved like an acid polysaccharide in immunoelectrophoresis It had a migration identical to that of glucosyl glycerol teichoic acid (poly B) in veronal buffer pH 8 8

Cell walls of *Staph epidermidis* 1254 gave one line against homologous anti bacterial serum which fused completely with the poly B line The antigen responsible for this line was removed from the walls only after prolonged extraction with 10 per cent TCA at elevated temperature It was sensitive to periodate and chromatographic examination of the hydrolysate of periodate treated cell wall showed that glucose had been oxidized

The cell walls from both micrococcal

TABLE 2 *Qualitative Paper Chromatographic Analyses of Sugars and Sugar Alcohols in Cell Walls*

	Staph epidermidis		*Micrococcus*	
	577	1254	congl 84	luteus 144
Glucose	+ +	+ +	+	+ + +
Galactose	+ +	—	+ +	—
Rhamnose	(+)	—	—	—
Mannose	—	—	(+)	(+)
Unidentified neutral sugars	—	—	—	+
Glucosamine	+ +	+ +	+ +	+ + +
Muramic acid	+ +	+ +	+ +	+ + +
Glycerol	+ +	+ +	+ +	+
Ribitol	—	—	—	—
Anhydroribitol	—	—	—	—

(+) to + + + Trace to strong reaction on chromatograms with the respective spray reagents
— No reaction

strains reacted only with homologous anti-bacterial sera to produce a single precipitin line in each instance The lines were due to different antigens and no correspondence with the reference systems was obtained Both precipitinogens were easily extracted by 5 per cent TCA and were sensitive to periodate

In the indirect haemagglutination test, NSE and TSE sensitized with extracts of *Staph epidermidis* strains (577 and 1254) were agglutinated in *Staph epidermidis* as well as *Staph aureus* anti bacterial sera, but not in the two *Micrococcus* sera The same result was obtained when *Staph epidermidis* cell walls were used as sensitizing agents, but the titres were generally lower When NSE or TSE were treated with extracts from the micrococcal strains weak agglutination was sometimes observed in *Staph epidermidis* and micrococcal anti bacterial sera Titres were never higher than 1/40 and were usually detected when sensitized NSE were used

All cell wall antisera agglutinated both NSE and TSE sensitized with bacterial extracts, and the 4 strains showed cross reactions Since this was in contrast to the results obtained with anti bacterial sera, serum from a rabbit injected only with complete *Freund's* adjuvant was tested and found to have a reactivity similar to that of the cell wall antisera

The cell wall antisera agglutinated homologous bacteria, but the titres were low However, absorption of anti bacterial sera with cell wall preparations of corresponding strains removed all agglutinins Cross-absorption studies indicated one or more shared agglutinogens between the 4 strains

DISCUSSION

The cell wall preparations may be considered as undigested since the possibility for degradation by autolytic enzymes during preparation was small

Since glycerol was the only sugar alcohol demonstrated, the 4 strains probably have wall teichoic acids of the glycerol type Assuming most of the phosphorus to be associated with teichoic acids the cell walls of the micrococcal strains apparently contain less teichoic acid than staphylococcal walls This is also indicated by the finding that the micrococcal cell walls had a lower content of glycerol

The results of the amino acid analyses and of the nitrogen determinations (Table 1), suggest that the cell walls of *Staph epidermidis* 1254 and *M luteus* 144 have a higher content of protein than the walls of the two other strains That *Staph epidermidis* 577 cell wall contained relatively large amounts

of amino acids not normally associated with mucopeptide indicates a higher content of other protein components

It has previously been shown that the cell walls of *M conglomeratus* contain small amounts of glycine (6, 33), and similar results have been obtained in the present experiments for *M conglomeratus* 84 In view of the large amounts of alanine, it would appear that in *M conglomeratus* 84 the cross-bridges of the mucopeptide are composed of alanine That large amounts of serine are present in the cell walls of the two *Staph epidermidis* strains agrees with the results of *Cummins* & *Harris* (6)

Hexoses were found in all cell wall preparations This contrasts with what has previously been found for the walls of *Staph aureus* (17), but the results are in agreement with regard to type and number of sugars found in the cell walls of micrococci (6) In *Staph epidermidis* 1254 glucose has been shown to be part of the teichoic acid (25), and in the other cell walls the hexoses are probably components of the teichoic acids

In serological tests, the cell walls of the *Staph epidermidis* strains were more active than those of the micrococcal strains One of the three precipitin lines obtained with *Staph epidermidis* 577 cell walls fused completely with the poly C line (26) That poly C appeared to cross react with antibodies to poly A indicates that poly A (β-N-acetylglucosaminyl ribitol teichoic acid) and poly C have N acetylglucosamine as a common antigenic determinant One of the two other precipitinogens demonstrated in cell walls from strain 577 was difficult to extract and resisted periodate oxidation However, it behaved like an acid polysaccharide in electrophoresis Teichoic acid structures stable to periodate have been isolated from micrococci (5), but have not been demonstrated in staphylococci

The three other cell wall preparations gave only one line against homologous anti bacterial sera, but no cross reactions were obtained The line given by *Staph epidermidis* 1254 cell walls was confluent with that of poly B, which has previously been characterized as a glycerol teichoic acid with α-linked glucose (9,25) The lines obtained with the two micrococcal cell walls could not be identified In contrast to an earlier report (29) no similarity with poly A or I_3 (α glucosaminyl glycerol) teichoic acid was observed

Lack of antibodies against teichoic acid in *Staph aureus* cell wall antisera has been assumed to be due to the immunization technique (17) Since similar results were obtained in the present experiments even though adjuvant was used, it seems more likely that structures present in cell walls had been altered or lost during preparation of the walls

In *Staph epidermidis* cell walls, both NSE and TSE sensitizing antigens were present and were found to be antigenically similar to corresponding antigens in *Staph aureus* In contrast, NSE and TSE treated with the micrococcal cell walls did not agglutinate in either staphylococcal or micrococcal antisera This indicates either a fundamental difference in the mucopeptide structure or lack of fragmentation of the micrococcal mucopeptide The weak reactions observed using extracts of micrococci for sensitization might point to the later explanation However, the finding that NSE and TSE sensitized with staphylococci did not agglutinate in micrococcal antisera is in agreement with the conclusion that these micrococcal strains lack NSE and TSE sensitizing antigens (29) Another possibility was revealed by the confusing results with cell wall antisera and serum from the rabbit injected with complete *Freund's* adjuvant only It is possible that the micrococcal extracts contained haptens which fix to NSE and TSE and are able to cross-react with antibodies elicited by antigens of the mycobacteria in the adjuvant

The content of bacterial agglutinogens in the cell wall preparations was small and the agglutinin response in rabbits was also low Similar findings have been reported for *Staph aureus* cell walls (23, 17) However, the results obtained after absorption of anti bacterial sera with the cell walls strongly

indicate that all agglutinogens present in the bacteria are also present in the cell walls

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IMMUNOCHEMICAL CHARACTERIZATION OF STAPHYLOCOCCAL AND MICROCOCCAL MUCOPEPTIDES

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Mucopeptides from one *Staphylococcus aureus* two *Staph epidermidis* and two *Micrococcus* strains have been prepared and examined for chemical composition and for serological activity The chemical analyses showed differences in the mucopeptide structures especially between staphylococci and micrococci some differences appearing to be related to variations in the inter peptide bridges No precipitation was observed but agglutination was obtained in antisera to whole bacteria as well as in antisera to mucopeptides Cross-absorption studies showed the presence of shared and specific antigenic determinants None of the mucopeptides sensitized erythrocytes for agglutination in antisera but the staphylococcal mucopeptides in contrast to the micrococcal induced the production of haemagglutinins in rabbits Antibodies to mucopeptides were found in human serum and colostrum The sera contained antibodies of the IgG class colostrum mostly IgA antibodies Sera with increased anti staphylolysin titres contained higher amounts of IgG than normal sera Fab but not Fc fragments of IgG were found to bind to mucopeptide and complexes of mucopeptide and IgG fixed complement Injection of mucopeptides into rabbit and human skin provoked dermonecrotic reactions

In a previous paper (12) the immunochemical properties of undigested cell walls of two *Staph epidermidis* and two micrococcal strains were studied The difference in serological properties between whole cells and their cell walls was quantitative rather than qualitative All strains seemed to share one or more agglutinogens but were found to differ in the type of precipitinogens The most pronounced difference between the cell walls of the staphylococcal and micrococcal strains was demonstrated by indirect haemagglutination of normal and tanned sheep erythrocytes (NSE and TSE) In this respect cell walls from *Staph epidermidis* were found to be antigenically similar to those of *Staph aureus* (14), whereas the micrococcal cell walls did not sensitize either NSE or TSE This was thought to be due to differences in the mucopeptide moieties

Chemical analyses for amino acids and sugars and examinations of serological reactivity suggested that the wall preparations contained other components in addition to mucopeptide and teichoic acid In the present experiments attempts have been made to prepare pure mucopeptides so that more detailed chemical and serological examinations of these structures could be made

Received 23 vi 71

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MATERIALS AND METHODS

Strains The *Staph epidermidis* strains (577 and 1254) and *Micrococcus* strains (*M conglomeratus* 84 and *M luteus* 144) were those used previously (12) *Staph aureus* strain 263 (16) was also used The bacteria were grown and harvested as before (12)

Preparation of mucopeptides Mucopeptides were prepared according to the method of *Park & Hancock* (25) The bacteria (5 g wet weight) were crushed in the X press and subsequently suspended in 500 ml of 5 per cent (w/v) cold trichloroacetic acid (TCA) and extracted at 0° C for 10 min. After centrifugation (4,000 *g* for 10 min was used throughout) the residue was homogenized and extracted with 500 ml of 75 per cent (v/v) ethanol for 10 min at room temperature and centrifuged The residue was re extracted with 500 ml of 5 per cent TCA for 10 min at 100° C, cooled and centrifuged After re suspension of the solid material in 200 ml of 0 05 M NH_4HCO_3, containing 0 005 M NH_4OH, 10 mg crystalline trypsin (Difco) were added and the mixture incubated at 37° C The digestion end point was determined spectrophotometrically (Unicam SP 800) at 700 nm. The insoluble material was then washed 3 times in distilled water, and if traces of teichoic acid were detected by serological testing, the material was further extracted with 5 per cent TCA for 24 hr at 20° C After 3 washings in distilled water, the preparations (TCA extracted mucopeptides) were dialysed and freeze dried

In some experiments TCA extracted mucopeptides were further treated with hot formamide according to *Fuller's* method (8) Mucopeptide (100 mg) was suspended in 40 ml formamide and digested at 160° C for 30 min in an oil bath, the suspension being agitated continuously The insoluble residue was washed 4 times in distilled water dialysed and freeze-dried

All mucopeptide preparations were examined by electron microscopy according to the earlier description (12)

Chemical examinations The methods used for quantitative determination of phosphorus, acid hydrolysis and circular paper chromatography were those described earlier (13) Amino acids in 6 N HCl hydrolysates of the mucopeptides were quantitated using a Perkin Elmer 900 gas-chromatograph (with digital integration) The N trifluoroacetyl n butyl esters of the amino acids were prepared as described in (9 26) and standard amino acid derivatives were purchased from Regis Chemical Comp The columns (200 cm × 2 mm) were filled with Tabsorb (Regis) 80–100 mesh, the flow rate of nitrogen being 25 ml per min

Enzyme treatment Mucopeptide which had been treated with ultrasonic (15 KC/s for 1 min in an MSE Mullard ultrasonic disintegrator) was suspended in buffer (3 ml), and the OD of the suspension adjusted to 1 0 at 725 nm Enzyme (0 2 mg) was added, and the digestion carried out at 37° C Digestions with lysozyme (eggwhite, cryst Koch Light) were performed in 0 1 M phosphate buffer, pH 6 2, and those with lysostaphin (gift from Dr Zygmunt, Mead Johnson, Ind, USA) in 0 01 M potassium phosphate buffer, pH 7 5 (32, 38) As test organisms, *M lysodeikticus* (NCTC 2665) was used for lysozyme and *Staph aureus* FDA 209 P for lysostaphin

Serological examinations The rabbit antisera to whole bacteria were those used previously (12) In addition, antisera to mucopeptides were prepared in rabbits given intravenous injections (24) of a total of 10 mg of TCA extracted mucopeptide previously treated with ultrasonic (20 KC/s for 15 min at 0° C)

Antisera specific for human IgG, IgA, and IgM were kindly furnished by Dr Bodil Larsen, The Gade Institute, and antisera specific for the Fab and Fc fragments of human IgG were purchased from Behringwerke AG Normal human sera, 10 sera with increased anti staphylolysin titres, Astaph sera, and human colostrum were obtained from Haukeland hospital, Bergen Fat free colostrum was acidified to pH 4 6 with N HCl and the precipitated casein was removed by centrifugation The neutralized supernatant was used for subsequent tests Purified Fab and Fc fragments of human IgG (Kabi) were prepared as described in (37)

Absorptions of sera with mucopeptides were carried out by adding 3 mg mucopeptide (homogenized by ultrasonic) to 0 25 ml of serum and incubating at 4° C for 24 hr The sensitized mucopeptides were washed 3 times in phosphate buffered saline (pH 7 2) and then suspended in 4 drops of HCl in saline (pH 2 0) The supernatant and 2 further washings were bulked and neutralized with 0 1 M trisbuffer, pH 8 0, in 0 15 M NaCl The concentrations of proteins (γ globulins) eluted from the washed mucopeptides were determined by the *Folin Ciocalteu test* (17) and by the *Mancini* agar precipitation test (21) Immunoelectrophoretic analyses were performed using an LKB 6800 A apparatus as described by the manufacturer (LKB Produkter), complement fixation on a micro scale as described in (20) using guinea pig serum as complement source, and double diffusion in agar, indirect haemagglutination slide and tube agglutinations as before (12)

Biological tests Rabbits were injected intradermally with 0 2 and 0 4 mg of disintegrated mucopeptide (15 KC/s for 10 min) in 0 2 ml of saline Each rabbit received 0 2 ml of sterile saline as a control One of the authors (S H) received an intradermal injection of 0 05 mg mucopeptide from *M luteus* 144 in 0 05 ml of saline and a control injection of 0 05 ml saline

TABLE 1 *Amino Acids (μ mole/100 mg) in TCA Extracted Staphylococcal and Micrococcal Mucopeptides*

	Staph aureus	*Staph epidermidis*		*Micrococcus*	
	263	577	1254	Congl 84	luteus 144
Alanine	122 9	127 7	139 3	312 5	125 7
Glutamic acid	71 0	82 2	86 6	86 2	111 3
Lysine	63 6	69 6	72 1	77 3	80 9
Glycine	296 3	248 4	241 2	12 3	84 3
Serine	3 5	9 8	39 6	0 7	2 0
Threonine	24 5	3 8	11 2	2 1	2 1
Leucine	12 9	14 2	20 7	11 2	11 9
Isoleucine	13 0	12 1	14 4	5 2	7 2
Aspartic acid	9 6	8 2	15 7	5 8	4 8
Valine	8 7	7 2	11 0	11 5	8 1
Proline	3 4	5 3	15 1	3 3	3 2
Phenylalanine	6 8	8 0	8 0	4 2	5 6

RESULTS

It was relatively easy to extract teichoic acid from the preparations of *Staph aureus and Micrococcus*, but prolonged extraction with TCA was necessary to remove all teichoic acid from the *Staph epidermidis* preparations The mucopeptides were shown by serological and chemical analyses to be free of teichoic acid components Only trace amounts of phosphorus were detected and paper chromatography of 3 N HCl hydrolysates revealed no sugar alcohols In the electron micrographs the mucopeptide particles appeared as amorphous spherical bodies

All preparations contained glucosamine and muramic acid The results of quantitative analyses for amino acids of TCA extracted mucopeptides are shown in Table 1 Amino acids in addition to those normally associated with mucopeptides were present Thus it appeared that pure mucopeptide preparations were not obtained Further extraction with hot formamide decreased the content of additional amino acids by 60–80 per cent

Table 2 *shows the molar ratios of the* amino acids lysine alanine, glutamic acid, glycine, and serine found in the different TCA extracted preparations The *Staph epidermidis* mucopeptides differed from those of *Staph aureus* in the content of glycine and serine However, this difference was small when compared to that between staphylococci and micrococci and the difference between the micrococcal mucopeptides The mucopeptide from *M conglomeratus* 84 contained only small amounts of glycine but a relatively high content of alanine In *M luteus* 144 mucopeptide the content of glycine was also low, compared to staphylococci, whereas the content of glutamic acid was higher

TABLE 2 *Molar Proportions of Some Amino Acids in Staphylococcal and Micrococcal TCA extracted Mucopeptides*

Mucopeptide	Amino acids Lys	Ala	Glu A	Gly	Ser
Staph aureus					
263	1	1 9	1 1	4 6	—
Staph epidermidis					
577	1	2 0	1 0	3 5	0 1
1254	1	2 3	1 3	4 0	0 3
Micrococcus					
congl 84	1	4 0	1 1	0 1	—
luteus 144	*1*	*1 4*	*1 4*	*1 1*	—

The effects of lysostaphin and lysozyme on the different mucopeptide preparations are illustrated in Fig 1 and 2, respectively

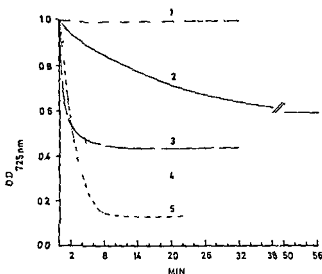


Fig 1 The effect of lysostaphin on the different mucopeptide preparations 1 *M conglomeratus* 84, 2 *M luteus* 144, 3 *Staph aureus* 263, 4 *Staph epidermidis* 577, and 5 *Staph epidermidis* 1254

The *Staph epidermidis* preparations were rapidly broken down by both enzymes So also was the *Staph aureus* mucopeptide, although to a lesser degree The *M luteus* 144 mucopeptide was very slowly affected by lysostaphin, but rapidly degraded by lysozyme Mucopeptide from *M conglomeratus* 84 was only partially sensitive to lysozyme and almost completely resistant to degradation by lysostaphin

No precipitation in agar gel was observed between mucopeptides and mucopeptide antisera or antisera to whole bacteria, and antisera to mucopeptides did not agglutinate whole bacteria However, antibodies to mucopeptides were demonstrated by agglutination of mucopeptide and by indirect haemagglutination The titres of agglutinating antibody *in preimmune sera* were *considerably* lower than those detected in immune sera and the agglutinates obtained with the latter were much stronger In mucopeptide agglutination tests cross-reactions were observed between all preparations and all sera but the titres were usually higher in homologous than in heterologous antisera Cross absorption studies indicated that all antigens of *Staph epidermidis* 1254 mucopeptide were present in the preparation from *Staph epidermidis* 577 and that additional antigenic determinants were present in the latter preparation There appeared to be greater differences between micrococcal mucopeptides than between staphylococcal mucopeptides

Ultrasonically-treated mucopeptides failed to sensitize NSE and TSE However, antisera to staphylococcal mucopeptides agglutinated both NSE and TSE sensitized with staphylococcal extracts, whereas the antisera to micrococcal muopeptides failed to agglutinate

Mucopeptides were agglutinated by all normal human sera, all Astaph -sera and by

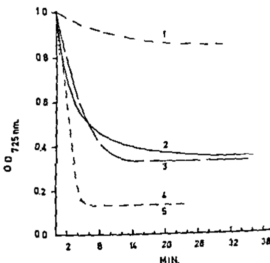


Fig 2 The effect of lysozyme on the different mucopeptide preparations 1 *M conglomeratus* 84 2 *M luteus* 144 3 *Staph aureus* 263 4 *Staph epidermidis* 577 and 5 *Staph epidermidis* 1254

human colostrum The strongest agglutinates and the highest titres were obtained with the Astaph sera The eluates obtained from *Staph aureus* mucopeptide sensitized by serum were found to contain IgG those obtained from the colostrum sensitized mucopeptide containing IgA together with small amounts of IgG It was difficult to elute all the γ-globulins from the strong agglutinates Thus reproducible results were not obtained by the *Folin Ciocalteau* and the *Mancini* tests In spite of this both tests showed that eluates obtained from mucopeptides sensitized with Astaph sera contained greater amounts of γ globulin (IgG) than the eluates obtained using normal human sera Only the Fab fragments from human IgG were found to bind to mucopeptide and complexes of mucopeptide and IgG fixed complement

Dermonecrotic lesions in rabbit skin were induced by intradermal injection of TCA extracted mucopeptides from *Staph epidermidis* or *Micrococcus* Similar results were obtained when formamide extracted mucopeptides were tested The mucopeptide from *M luteus* 144 was found to induce dermonecrotic lesion in human skin

DISCUSSION

Since extraction with hot formamide failed to remove the amino acids not usually associated with mucopeptides chemical and serological examinations were mostly performed with TCA extracted preparations

The results of serological and chromatographic analyses show the mucopeptide preparations isolated in the present experiments to be free of teichoic acids even though trace amounts of phosphorus were detected in all preparations Small amounts of phosphorus are usually found in mucopeptide preparations (4 18 7 28) and may be due to the presence of phosphorylated amino sugars (28) That the teichoic acids of *Staph epidermidis* strains were difficult to extract with TCA is in conformity with a previous observation (7)

Variations in the molar proportions of the typical mucopeptide amino acids (*viz* Ala Lys GluA and Gly) were observed in the different staphylococcal mucopeptides It has been reported that glycine is often replaced by serine in mucopeptides from *Staph epidermidis* (6 10 33) and the occurrence of 4 types of penta peptide brigdes has been reported in *Staph epidermidis* (10) More over about one third of the peptide subunits of the staphylococcal peptide are pentapeptides the COOH terminal D alanine of the muramyl pentapeptide precursor not having been split off (33) In the micrococcal mucopeptides quite different molar ratios of component amino acids were found in agreement with an earlier report (31) The glycine content was low especially for *M conglomeratus* 84 mucopeptide This mucopeptide had a high content of alanine suggesting a peptide bridge similar to that found in *M roseus* R 27 (10) Relatively high amounts of glutamic acid were found in the mucopeptide from *M luteus* 144 suggesting a similarity with the mucopeptides from *M citreus* and *M varians* (31) and it is possible that the higher glutamic acid content is due to the replacement of glycine by glutamic acid The latter has been reported in another strain of *M luteus* (23)

That the mucopeptide from *M conglomeratus* 84 was only very slightly affected by lysostaphin and the preparation from *M luteus* 144 only slowly degraded by this enzyme is consistent with the small amounts of glycine found in these preparations Lysostaphin is known to be an endopeptidase acting specifically on glycyl glycine linkages (32 5) It is possible that the partial degradation observed was due to the presence of other enzymes in the lysostaphin preparation Endo acetyl glucosaminidase and acetylmuramyl L alanine amidase activities have been found in at least one preparation of lysostaphin (40)

Differences in the sugar chains of the mucopeptides from *M conglomeratus* 84 and *M luteus* 144 would account for the results obtained when lysozyme (endoacetyl muramidase) was incubated with these preparations

(Fig 2) Variations in acetylation of amino sugars have been found to influence the susceptibility of mucopeptides to degradation by lysozyme (11, 30) and the nature of the sugar linkages is also important in this regard (31, 30)

In contrast to the mucopeptides from the staphylococci used in the present experiments, the micrococcal mucopetides did not elicit the synthesis of haemagglutinins in rabbits However, agglutination of micrococcal mucopeptides was obtained wih antisera to micrococcal and staphylococcal mucopeptides in considerably higher titres than with the preimmune sera This finding indicates antigenic similarity of the micrococcal and staphylococcal mucopeptides That agglutination titres were highest in homologous anti mucopeptide sera, in conjunction with the results from cross-absorption experiments, suggest specific determinants in addition to those shared by micrococcal mucopeptides The *Staph epidermidis* mucopeptides were found to be antigenically similar However, mucopeptide of strain 577 contained distinctive determinants not associated with the preparation from strain 1254

The role of structures other than mucopeptide possibly present in the preparations (cfr the amino acid composition) is obscure In this connection it is worthwhile to note that antisera to mucopeptides did not agglutinate whole bacteria

That IgG from normal human and human Astaph sera reacts with mucopeptide is consistent with an earlier report that the Cohn fraction II of normal sera reacts with bacterial mucopeptides (1) In view of the finding that Fab but not Fc fragments from normal IgG became fixed to mucopeptide it seems that the reaction between IgG and mucopeptide is a specific antibody antigen reaction This is also indicated by the finding that Astaph sera contained higher amounts of specific IgG than normal human serum Of special interest is the finding that human colostrum contained antibodies of the IgA type capable of reacting with mucopeptide The presence of IgA in human colostrum specific for various microbes has been reported previously (3, 15, 22), and it is possible that the presence of such antibodies is of importance in protecting the mammary gland from microbial infections (39, 22)

Intradermal injections of bacterial cell walls and mucopeptide preparations are known to induce dermonecrotic reactions in various animal species (27, 35, 1, 19, 29, 36) However, the mechanism of such reactions is not clear It is possible that mucopeptides have a direct toxic effect (34), but it is considered more likely that antibody-antigen complexes bind complement *in vivo*, in view of the finding that complexes of IgG and mucopeptide fix complement *in vitro* The latter possibility is supported by histological examinations (2, 34)

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ANTIGENIC STUDIES ON *STAPHYLOCOCCUS EPIDERMIDIS*

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An antigenic analysis has been performed of 48 strains of *Staphylococcus epidermidis*, mostly belonging to biochemical type 1 Rabbit preimmune sera were found to contain shared natural antibodies in low titres against *S epidermidis* and *S aureus* Cross agglutination and absorption experiments showed that the two species share antigens to a considerable degree After removal of the shared antibodies by absorption, several specific *S epidermidis* antibodies were registered Glycerol α glucosyl teichoic acid (polysaccharide B) was demonstrated in 67 per cent of the *S epidermidis* strains on double diffusion in agar gel An unknown precipitinogen was demonstrated in 37 strains Serologic examination is recommended in the evaluation of coagulase negative staphylococci isolated from infections

The wall teichoic acid of *Staphylococcus epidermidis* is of the glycerol type with glucosyl residues (2 3, 14, 17) Double diffusion in agar shows that the teichoic acid has two serologic specificities, depending upon the position of the glucosyl residues (α or β) (15, 17, 3, 22) *S epidermidis* thus differs from *S aureus* in the type of its wall teichoic acid, the latter being of the ribitol type with N acetylglucosaminyl residues, and in the serologic properties of the teichoic acid Cross agglutinations between the two species occur regularly (23) The shared agglutinogens, and the specific *S epidermidis* agglutinogens, presumably present, are completely unknown, whereas the specific *S aureus* agglutinogens have been extensively investigated (19)

Considering the ubiquity of *S epidermidis*, its increasing significance in human and animal infections, even in serious infections such as septicaemia and endocarditis (25, 24), and the problems often encountered in the classification of coagulase negative, pathogenic strains (26), a better knowledge of the antigens of this organism seems to be important The aim of the present study was to acquire a general picture of the antigens of *S epidermidis* as a basis for more detailed continued investigations

Received 15 vi 71

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MATERIALS AND METHODS

Strains All the 48 strains of *S epidermidis* examined serologically produced catalase, produced acid from glucose anaerobically (27) and were coagulase negative When the strains were examined biochemically according to *Baird Parker* (1) 39 strains were found to belong to type 1

The origin of the strains of *S epidermidis* used for the production of immune sera and in the absorption experiments was

Routine strains from the Institute 289, 456, 630, 785, 1188, 12105 12142

Losnegard & Oeding (14) 1254

Dr M Kocur Czechoslovak Collection of

Microorganisms, Brno, Czechoslovakia 577, 33363, 50 (*Micrococcus violagabriellae*), 2368 (*M hyicus*) The two latter strains were classified by us as *S epidermidis* This is in accordance with the data given in (28)

Dr J Maclean Smith, State University of Iowa, Iowa City, USA Oxenrider, Stubbs

The majority of the remaining strains of *S epidermidis* were obtained from the diagnostic laboratory of the Institute, whereas a few strains were derived from type collections

Eight *S aureus* type strains (8) and 8 *Micrococcus* strains (20) were included in the investigation

Sera Preimmune sera and immune sera were obtained from the Institute s breed of New Zealand white rabbits Immune sera where produced by intravenous injections of formalin killed bacteria (18) of the following *S epidermidis* strains 456, 1188, 12142, 577, Oxenrider 1254, 50 (viola gabr), 2368 (hyicus) In addition some *S aureus* and *Micrococcus* immune sera were used *S aureus* factor sera were produced according to the method of *Oeding*

Agglutination Agglutination was carried out on slides using live bacteria For technical details, see (8, 9)

Double diffusion in agar The technique was as described in (10) Thick suspensions of 18 hr live nutrient agar cultures of the strains were tested against undiluted immune sera Polysaccharide A (7) polysaccharide 263 (12) protein A (5) and antigen D (13) from *S aureus* polysaccharide B from *S epidermidis* and polysaccharide C from *Micrococcus* (15) were included as references

Indirect haemagglutination Normal and tanned sheep erythrocytes were sensitized with phosphate buffered saline extracts of the bacteria and tested for agglutination in dilutions of the immune sera (21) The reactions were incubated 30 min at 37° C and then overnight at room temperature and read 1 as a pattern 2 after gentle shaking 3 15 min after vigorous shaking

RESULTS

Agglutination in Rabbit Preimmune Sera

Fourteen selected *S epidermidis* strains were tested in 3 rabbit preimmune sera Eight, 6 and 3 strains agglutinated in each of the undiluted sera Only 1 strain agglutinated in two of the sera diluted 1 10

Two preimmune sera were chosen in which two *S aureus* strains (17 A Cowan I) and two *S epidermidis* strains (Stubbs, 12105) agglutinated in titres above 1 10 The sera were absorbed with each strain The *S aureus* strains removed all natural antibodies against both the aureus and the epidermidis strains, whereas after absorption with the epidermidis strains the sera still agglutinated the aureus strains

Agglutination in S aureus and Micrococcus Immune Sera

All 48 *S epidermidis* strains were tested for agglutination in dilutions of 4 *S aureus* immune sera (Table 1) Two of the sera (17 A, 1503) had rather strong antibodies against the majority of the *S epidermidis* strains The two other sera (Cowan I, Wood 46) usually agglutinated the *S epidermidis* strains in low titres

The *S epidermidis* strains were then tested for the presence of specific *S aureus* agglutinogens in the 13 *S aureus* factor sera Many strains agglutinated in the factor sera *n*, *e*, and h_1, viz 29, 21, and 15 strains respectively Only scattered reactions were observed in 4 other factor sera and no reaction in the remaining sera Ten strains did not agglutinate in any serum The 21 strains which agglutinated in the *e* serum were also positive in the *n* serum These two factor sera are produced by absorption of the same immune serum which indicated that the agglutination might be caused not by the specific *S aureus* serum factors but by natural antibodies not removed by the absorption This was confirmed on absorption of the factor sera *n*, *e*, and h_1 with two of the agglutinating *S epidermidis* strains The agglutination of the epidermidis strains was now abolished, whereas anti *n* *e*, and h_1 were still present

On agglutination of the 48 *S epidermidis* strains in 4 *Micrococcus* immune sera, the majority were found to be positive in the undiluted sera, whereas only a few strains agglutinated in serum dilution 1 10

Agglutination in S epidermidis Immune Sera

All strains of *S epidermidis*, 8 strains of *S aureus*, and 8 strains of *Micrococcus* were tested on agglutination in dilutions of 8 *S epidermidis* immune sera Three sera (2368

TABLE 1 *Agglutination of 48* S epidermidis *Strains in 4* S aureus *Immune Sera*

S aureus immune serum	No of strains agglutinating in serum dilution 1	10	20	40	80	160	320	640	1280	2560
17 A	41	44	44	37	13	8	1	0	x	
1503	45	45	45	44	30	11	1	0		x
Cowan I	31	12	11	8	4	2	0			x
Wood 46	38	2	1	1	0				x	

Titres given as reciprocal values
x homologous titre

(hyicus), 577, Oxenrider) agglutinated few strains in titres over 1 10, whereas 5 sera agglutinated the majority of the strains of all three groups in relatively high titres Table 2 gives the reactions in one of the strongly reacting sera The reactions indicated that the majority of the antibodies of the latter sera were against antigens shared by the *S epidermidis* and *S aureus* strains, and in part also by the *Micrococcus* strains, whereas specific *S epidermidis* antibodies seemed to be present in smaller amounts In sera 2368 (hyicus), 577, and Oxenrider there seemed to be an excess of specific *S epidermidis* antibodies, although even here cross reacting antibodies were detected

Absorptions of S epidermidis Immune Sera

To remove shared antibodies, each of the 8 *S epidermidis* immune sera was first absorbed with *S aureus* strains 17 A and 1503, which had been shown to be rich in cross reacting antigens (see Table 1) None of the absorbed sera agglutinated a set of 6 *S aureus* strains The sera were then absorbed successively with *S epidermidis* strains until exhaustion Thirteen selected *S epidermidis* strains, including the sera strains, were tested in the sera before and after absorption

Two sera (2368 (hyicus), 577) reacted only with the homologous strain after absorption with the two *S aureus* strains Each of the strains 2368 (hyicus) and 577 consequently seems to have one agglutinogen specific to that strain

After absorption with the two *S aureus* strains, serum 1254 reacted with the homologous strain, strain 630, and, weakly, with two additional strains (Table 3) After absorption with 630 bacteria also, only the homologous strain agglutinated Thus strains 1254 and 630 share one specific agglutinogen, 1254 having an additional specific agglutinogen

After absorption with the two *S aureus* strains and *S epidermidis* 630, four sera (12142 456, 1188, 50 (violagabr)) agglutinated the same 7 strains (Table 4) Any of these strains was able to exhaust all the 4 sera Thus these sera have antibodies against

TABLE 2 *Agglutination of* S epidermidis, S aureus *and* Micrococcus *Strains in* S epidermidis *12142 Immune Serum*

Strains tested		No of strains agglutinating in serum dilution 1	10	20	40	80	160	320	640	1280
S epidermidis	48	45	45	45	45	44	39	33	24	1x
S aureus	8	8	8	8	8	8	8	4	2	1
Micrococcus	8	8	8	4	3	3	0			

Titres given as reciprocal values
x homologous strain

TABLE 6 *Precipitinogens Demonstrated in 48 S epidermidis Strains on Double Diffusion in Agar*

	Polysacch A	Polysacch 263	Polysacch B	Polysacch C	Protein A	Antigen D
No of strains producing precipitin line	6	0	32	1	0	25
In per cent	12	0	67	2	0	52

The cross agglutination and absorption experiments with immune sera confirmed that *S epidermidis* and *S aureus* share antigens to a considerable degree Some sera have rather high titres of cross reacting antibodies whereas in others the titres are relatively low (Table 1 2) There are at least two, probably several specificities among the shared antigens (Table 3–5) Similar results were reached by indirect haemagglutination of normal and tanned sheep cells Cross agglutinations between *S aureus* and *S epidermidis* are well known (23, 16)

The results further confirm the demonstration by Oeding (20) of a weak sharing of antigens between staphylococci and micrococci Due to the generally quite low titres it could not easily be decided whether these cross reactions were caused by natural antibodies or by immune antibodies (Table 2)

After removal of the shared antibodies on absorption of the *S epidermidis* immune sera with bacteria of the selected *S aureus* strains 1503 and 17 A it was quite simple to identify the remaining antibodies by further absorption with *S epidermidis* strains Several specific *S epidermidis* antibodies were registered some of which reacted with only one test strain whereas others reacted with several strains The results did not indicate the existence of one specific *S epidermidis* agglutinogen shared by all the strains

S aureus and *S epidermidis* apparently have a common basic structure of agglutinogens, which is to some extent shared also by micrococci In addition each species has varying amounts of specific agglutinogens which have been thoroughly investigated in *S aureus* The present results indicate that the number and amounts of specific agglutinogens may be smaller in *S epidermidis* than in *S aureus*

If *S epidermidis* strains agglutinate in the factor sera used for the typing of *S aureus* strains, the natural conclusion will be that these antigens are not specific to *S aureus* but are present also in *S epidermidis* strains (6) In the present investigation several *S epidermidis* strains agglutinated in certain factor sera As the antibodies agglutinating the *S epidermidis* strains, but not the specific *S aureus* antibodies, were removed on absorption with *S epidermidis*, it was concluded that *S epidermidis* does not possess the specific *S aureus* antigens The agglutination in certain *S aureus* factor sera was explained by the presence of natural antibodies which had not been removed by the *S aureus* strains used for absorption This conclusion was also reached by *Oeding* (20) *Haukenes* (9) warned against the production of *S aureus* factor sera without proper measures to ensure the removal of reacting natural antibodies

The presence of glycerol α glucosyl teichoic acid (polysaccharide B) was demonstrated serologically in 67 per cent of the *S epidermidis* strains on double diffusion in agar gel This agrees with the conception that glycerol glucosyl teichoic acid is characteristic of *S epidermidis* (2, 3 14, 17) The sugar can be in α or in β position (3) As no serologic reference system was available for the demonstration of the β linked antigenic determinant it is probable that some of the strains which gave no precipitin reaction with the α linked determinant may have the β linked teichoic acid

The teichoic acid of *S aureus* is of the

ribitol type with N-acetylglucosaminyl residues An antigen cross reacting with antibodies against its β linked determinant (polysaccharide A) was demonstrated in 6 *S epidermidis* strains Also *Haukenes* (7) and *Losnegard & Oeding* (15) reported on some *S epidermidis* strains giving the polysaccharide A line The significance of this observation is not clear

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It seems to us that a serologic investigation should be performed and given considerable weight in the evaluation of coagulase negative staphylococci isolated from infections *S aureus* and *S epidermidis* each has a teichoic acid, the two antigenic determinants of which can relatively easily be demonstrated on double diffusion in agar Also protein A which is present only in *S aureus*, can easily be demonstrated by this method Further, the presence of specific *S aureus* agglutinogens shows that a strain belongs to *S aureus*

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If *S epidermidis* strains agglutinate in the factor sera used for the typing of *S aureus* strains, the natural conclusion will be that these antigens are not specific to *S aureus* but are present also in S *epidermidis* strains (6) In the present investigation several *S epidermidis* strains agglutinated in certain factor sera As the antibodies agglutinating the *S epidermidis* strains, but not the specific *S aureus* antibodies, were removed on absorption with *S epidermidis*, it was concluded that *S epidermidis* does not possess the specific *S aureus* antigens The agglutination in certain *S aureus* factor sera was explained by the presence of natural antibodies which had not been removed by the *S aureus* strains used for absorption This conclusion was also reached by *Oeding* (20) *Haukenes* (9) warned against the production of *S aureus* factor sera without proper measures to ensure the removal of reacting natural antibodies

The presence of glycerol α glucosyl teichoic acid (polysaccharide B) was demonstrated serologically in 67 per cent of the *S epidermidis* strains on double diffusion in agar gel This agrees with the conception that glycerol glucosyl teichoic acid is characteristic of *S epidermidis* (2 3, 14, 17) The sugar can be in α or in β position (3) As no serologic reference system was available for the demonstration of the β linked antigenic determinant it is probable that some of the strains which gave no precipitin reaction with the α linked determinant may have the β linked teichoic acid

The teichoic acid of *S aureus* is of the

ribitol type with N-acetylglucosaminyl residues An antigen cross reacting with antibodies against its β linked determinant (polysaccharide A) was demonstrated in 6 *S epidermidis* strains Also *Haukenes* (7) and *Losnegard & Oeding* (15) reported on some *S epidermidis* strains giving the polysaccharide A line The significance of this observation is not clear

None of the *S epidermidis* strains in the present material had the *S aureus* antigen protein A, whereas 52 per cent had antigen D, which has been reported to be present both in *S aureus* and in *S epidermidis* strains (13)

A precipitin line common to 37 of the 48 *S epidermidis* strains was demonstrated The nature of the antigen involved is unknown but it is probably not related to a teichoic acid

It seems to us that a serologic investigation should be performed and given considerable weight in the evaluation of coagulase negative staphylococci isolated from infections *S aureus* and *S epidermidis* each has a teichoic acid, the two antigenic determinants of which can relatively easily be demonstrated on double diffusion in agar Also protein A which is present only in *S aureus*, can easily be demonstrated by this method Further, the presence of specific *S aureus* agglutinogens shows that a strain belongs to *S aureus*

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TABLE 6 *Precipitinogens Demonstrated in 48 S epidermidis Strains on Double D*

	Polysacch A	Polysacch 263	Polysacch B	Polysacch C
No of strains producing precipitin line	6	0	32	
In per cent	12	0		

The cross agglutination and absorptio experiments with immune sera confii that *S epidermidis* and *S aureus* share ant gens to a considerable degree Some sera have rather high titres of cross reacting anti bodies whereas in others the titres are relatively low (Table 1, 2) There are at least two, probably several specificities among the shared antigens (Table 3–5) *Similar results were reached by indirect hae magglutination of normal and tanned sheep* cells Cross agglutinations between *S aureus* and *S epidermidis* are well known (23 16)

The results further confirm the demonstra tion by Oeding (20) of a weak sharing of antigens between staphylococci and micro cocci Due to the generally quite low titres it could not easily be decided whether these cross reactions were caused by natural anti bodies or by immune antibodies Table 2)

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A POLYSAC(, IDE ANTIGEN OF AN ANAERO ( ) ! FILAMENTOUS MICROO! , EUBACTERIUM *SABURREU'* , NING HEPTOSE AND o-ACETY CONSTITUENTS

1 *Isolation* ri ation of the Antigen

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Laborator Depa tn nt of Microbiology,
The Gade Institu and Medi ne University of Bergen
rway

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Morphological, physiological and serological properties of an anaerobic filamentous micro organism isolated from the human oral cavity have been reported (4) The microorganism proved to be identical to a filamentous bacterium described by *Theilade & Gilmour* in 1961 (17) The organism was named *Catena bacterium saburreum* by *Prevot* (13) and will appear in the 8th edition of Bergey s Manual of Determinative Bacteriology under the name *Eubacterium saburreum* (16)

Cell walls of the filamentous micro organism contained approximately 50 per cent protein 20 per cent lipid and 20 per cent neutral sugar (5) A characteristic sug component was an aldoheptose, tentative identified as D glycero D galacto-heptos The heptose was removed quantitatively fro cell walls or whole cells by digestion wit trypsin which also removed considerab amounts of protein and lipid The fil mentous organism exhibited a multilayere cell wall the outer parts of which disap peared following trypsin digestion (6)

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A POLYSACCHARIDE ANTIGEN OF AN ANAEROBIC ORAL FILAMENTOUS MICROORGANISM (*EUBACTERIUM SABURREUM*) CONTAINING HEPTOSE AND o-ACETYL AS MAIN CONSTITUENTS

1 *Isolation and Chemical Characterization of the Antigen*

TOR HOFSTAD

Laboratory of Oral Microbiology Department of Microbiology
The Gade Institute Schools of Dentistry and Medicine University of Bergen
Bergen Norway

A serologically highly active polysaccharide has been isolated from the filamentous oral organism strain I 44 The isolation procedure entailed the digestion of whole, acetone dried cells with trypsin followed by fractionation of the supernatant fluid on columns of Sephadex G 75 and DEAE cellulose Heptose and 0 acetyl accounted for approximately 90 per cent of the purified antigen preparations All preparations contained small amounts of protein Lipid or hexosamine were not found

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pronounced specificty than endoantigens detected by precipitinogenic determinations However, more penetrating analyses of precipitinogens employing a complete pattern might lead to a more advanced differentiation especially as it gives a more complete picture of the antigenic mosaic

* This study was supported by grants from *WHO and Ellen, Walter and Lennart Hesselman's Foundation for Scientific Research*

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MATERIALS AND METHODS

The filamentous organism, strain L44, was cultivated in enriched nutrient broth (4). The harvested and washed bacteria were kept frozen until used.

Extraction and Purification Methods

Acetone dried whole cells were digested with crystalline trypsin (Trypsin 1 250 Difco), freshly prepared in 0 02 M tris (hydroxymethyl)aminomethane buffer containing 0 05 M $CaCl_2$, pH 7 8, using 0 02 mg of the trypsin preparation per mg whole cells. Sephadex G 75 (AB Pharmacia Uppsala Sweden) was used for gel filtration. The columns were stabilized and eluted with 0 02 M phosphate buffer pH 7 4 containing 0 001 M EDTA and 0 02 per cent sodium azide. Ion exchange chromatography was carried out on columns of DEAE cellulose (DEAE SS, Serva Heidelberg Germany). Elution was performed with 0 02 M phosphate buffer pH 7 4 or with a NaCl gradient in the same buffer.

Paper chromatography. Acid hydrolysis was done in sealed tubes at 100° C with N H_2SO_4 for 4 hr or with 3 N HCl for 3 hr. The H_2SO_4 hydrolysates were neutralized by passage through a column of Dowex 1 in the formate form. Acid was removed from HCl hydrolysates by evaporation over NaOH pellets. Circular paper chromatography was carried out with *n* butanol pyridine water (6 4 3). Sugars were stained with silver nitrate or aniline hydrogen phthalate. Hexosamines were also sought with the Elson Morgan reagent of *Partridge* (12).

Chemical analyses. Nitrogen was determined by the micro-Kjeldahl method (8). Samples were digested for 4 hr. Protein was measured by the Folin Ciocalteau phenol method (10) with bovine serum albumin as standard. The sulphuric acid cysteine reaction of *Dische* (2) was used for determination of heptose. D glycero D galacto heptose served as standard. O acetyl groups were determined by alkaline hydroxylamine as described by *Hestrin* (3) with acetylcholin HCl as standard. Acethydroxamic acid was identified by thin layer chromatography. Cellulose plates (Eastman Chromagram Sheets) were run with ethyl acetate pyridine water (2 1 2), dried and sprayed with acid ferric chloride (3).

Serological Methods

Ring test precipitation was carried out in capillary tubes with undiluted rabbit antiserum prepared against whole microbes. Difco Special Agar Noble (1 per cent) in saline was used for gel diffusion tests.

EXPERIMENTS AND RESULTS

Preparation of the antigen. In pilot experiments different methods for extraction of antigenic materials from whole cells were tried, including extraction with cold 5 per cent trichloroacetic acid, trypsin digestion and treatment with phenol water (20). Digestion with trypsin was approximately twice as effective as extraction with trichloroacetic acid. After extraction with phenol-water ring test positive material was recovered from the phenol phase but in low yields.

The dialysed supernatant following digestion with trypsin gave 2 agar precipitation lines against antiserum L44: a heavy line present in high dilutions of the extracts (the polysaccharide antigen) and 1 weak line (contaminating precipitinogen). Based on preliminary fractionation experiments a simple method comprising gel filtration on Sephadex G-75 and ion exchange chromatography with DEAE cellulose was adopted for isolation of the polysaccharide antigen.

In a representative experiment (Table 1, Batch 2) 2 g acetone dried whole bacteria

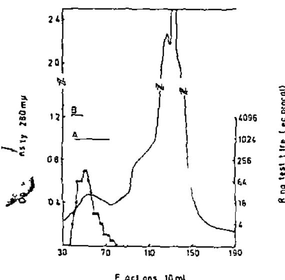


Fig 1. Gel filtration on Sephadex G 75 of supernatant fluid from trypsin digestion of 2 g acetone dried L44 bacteria. A Polysaccharide antigen. B Blue dextran (separate run). Dotted line Contaminating precipitinogen. ●——● Ring test fractions against antiserum L44.

were digested with 40 mg trypsin in 500 ml buffer at 37° C for 20 hr After centrifugation (10 000×g 15 min) the supernatant fluid was dialysed against tap water overnight and the volume reduced to approximately 25 ml by evaporation *in vacuo* at 40° C The concentrated digest, which formed a slightly opalescent suspension, was applied to a column of Sephadex G 75, 4 5× 75 cm and 10 ml fractions were eluted at a flow rate of 25 ml/hr (Fig 1) The fractions were examined by ring test precipitation and double diffusion in agar against antiserum L44 The polysaccharide antigen appeared with the void volume and in subsequent fractions up to fraction no 73, well separated from the bulk of ultraviolet light absorbing material The fractions containing the polysaccharide antigen (fraction 38–73) were pooled and dialysed against tap water overnight After reduction of the volume to approximately 25 ml by evaporation *in vacuo* the material was applied to a column of DEAE cellulose, 2×24 cm (Fig 2) Elution was performed with phosphate buffer at a flow rate of 10 ml/hr The bulk of the polysaccharide antigen passed straight through the column (fractions 7–10) Smaller amounts of the specific antigen were slightly retarded by the resin together with contaminating precipitinogen (fractions 11–13) Small amounts of the antigen as judged by ring test titre, were also eluted with 0 05 to 0 2 M NaCl in the buffer Fractions 7–10 were pooled, dialysed against tap water for 4 days and finally against distilled water The yield of lyophilized purified polysaccharide antigen was 40 mg An 0 1 per cent solution of the purified polysaccharide antigen in saline gave ring test precipitation in dilutions up to 1/1024 with undiluted antiserum L44 The corresponding figure for the freeze dried supernatant after tryptic digestion was 1/64 Based on serological activity per unit dry weight a 16 fold purification thus had been achieved

The amount of purified polysaccharide antigen obtained from 2 g of acetone dried whole L44 cells varied in different exper

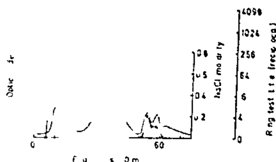


Fig 2 Chromatography of serologically active material recovered from Sephadex G 75 on DEAE cellulose at pH 7 4 Polysaccharide antigen Dotted line Contaminating precipitinogen ● — ● Ring test titre of fractions against antiserum L44

iments from 20 to 70 mg The critical step in the purification procedure was the ion exchange chromatography on columns of DEAE cellulose Sometimes the first two or three antigen containing 10 ml fractions only were free of contaminating precipitinogen In the most successful experiment (Table 1, Batch 1) uncontaminated polysaccharide antigen was eluted in seven 10 ml fractions The yield of purified antigen also depended on the trypsin digestion which released somewhat variable amounts of antigen from the acetone dried cells

Chemical Composition of the Polysaccharide Antigen

Paper chromatography of hydrochloric and sulphuric acid hydrolysates of all antigen preparations revealed one predominant sugar component with the same mobility and colour on aniline hydrogen phthalate treated chromatograms as D glycero D galacto heptose Some of the preparations (among them Batch 1 and Batch 2, Table 1) also contained trace amounts of glucose Barely visible spots which moved like rhamnose and xylose in the developing system used, were detected when large amounts of the h olyzed preparations

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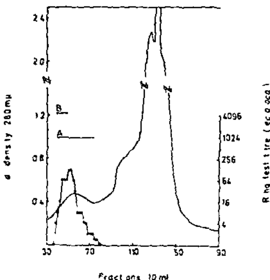


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The amount of purified polysaccharide antigen obtained from 2 g of acetone dried whole L44 cells varied in different exper-

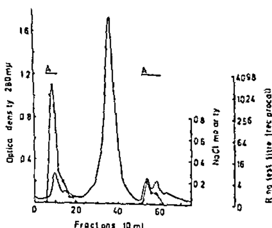


Fig 2 Chromatography of serologically active material recovered from Sephadex G 75 on DEAE-cellulose at pH 7 4 A Polysaccharide antigen Dotted line Contaminating precipitinogen ●———● Ring test titre of fractions against antiserum L44

iments from 20 to 70 mg The critical step in the purification procedure was the ion exchange chromatography on columns of DEAE cellulose Sometimes the first two or three antigen containing 10 ml fractions only were free of contaminating precipitinogen In the most successful experiment (Table 1, Batch 1) uncontaminated polysaccharide antigen was eluted in seven 10 ml fractions The yield of purified antigen also depended on the trypsin digestion which released somewhat variable amounts of antigen from the acetone dried cells

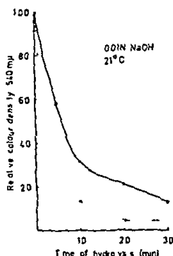
Chemical Composition of the Polysaccharide Antigen

Paper chromatography of hydrochloric and sulphuric acid hydrolysates of all antigen preparations revealed one predominant sugar component with the same mobility and colour on aniline hydrogen phthalate treated chromatograms as D glycero-D galacto hepte Some of the preparations (among them B 1 and Batch 2, Table 1) also contained tr amounts of glucose Barely visible spots wl moved like rhamnose and xylose in developing system used, were detected large amounts of the hydrolyzed prep

ıBI E 1 *Serological Activity and Chemical Composition of Three Batches of Polysaccharide ı gen Isolated from 2000 mg Portions of Acetone Dried Whole Cells of* Eubacterium saburreum *Strain* L44

	Yield mg	Serological activity[a]	Protein Per cent	Nitrogen Per cent	Phosphorus Per cent	Heptose Per cent	Heptose μ moles/mg[b]	O acetyl μ moles/mg
tch 1	70	1/2048	4 5	0 8	0 2	86	4 1	3 3
ıch 2	40	1/1024	7 2	1 4	0 1	81	3 9	2 5
tch 3	20	1/1024	5 6	0 8	0 1	92	4 4	3 0

Highest dilut on (twofold) of 1 mg/ml giving positive ring test against antiserum L44
Calculated



g 3 Colour formation in the hydrolyxamine ric chloride reaction of Hestrin (3) after expo- re of polysaccharide antigen (unbroken line) and etylcholin chloride (broken line) to 0 01 N dium hydroxide

ed to the paper Glucosamine or sugars could not be demonstra ... chloric acid hydrolysates by chromatography or in the reaction of *ondle & Morgan* (14) None of the prepa tions contained sugar alcohols or material cting in the malonealdehyde thiobarbituric d reaction of *Weissbach & Hurwitz* (19) *h her* fatty acids were not detected by thin r chromatography of an ether extracted lkaline hydolysate

The results of quantitative chemical ana ses of three preparations of the polysaccha ide antigen have been compiled in Table 1

Different methods were used for identification of the alkali labile O esters as O acetyl Fig 3 shows the rate of alkaline hydrolysis of the hydroxylamine reactive groups of the polysaccharide antigen Incubation of the antigen with 0 01 N NaOH for 5 min at room temperature caused an approximately 50 per cent reduction in reactive 0 esters as measured by formation of hydroxamic acid The reactive 0 esters were stable in 1 per cent acetic acid at 100° C for at least 2 hrs Finally equal volumes of an aqueous solution of polysaccharide antigen and the alkaline hydroxylamine reagent were mixed and allowed to stand at room temperature for 5 min The neutralized reaction mixture was evaporated to dryness *in vacuo*, redissolved in water and subjected to thin layer chromatography Spraying with acid ferric chloride revealed one spot with an Rf value of 0 6 A spot with the same Rf value as acethydroxamic acid was obtained when acetylcholine chloride was treated in the same way

DISCUSSION

The procedures used for isolation of the highly active polysaccharide antigen from the filamentous organism *Eubacterium saburreum* strain L44 gave reproducible results in repeated experiments Judged from serological activity in ring test precipitation nearly all precipitating antigen in the supernatant fluid after trypsin digestion of ace

dried L44 whole cells was recovered as
lande antigen Batch 3 (Table 1)
n exception

e results of the qualitative and quantita
hemical examinations show that the
c antigen contains heptose and 0 ace-
major constituents N acetyl groups do
act with alkaline hydroxylamine under
nd tions used (3) The stability of the
echaride antigen to acid precludes the
e of acylphosphate

e molar ratio of heptose to 0-acetyl
from one preparation to another
3 (Table 1), which showed a molar
of these main constituents of approx
s 1 2 1, was examined for 0 acetyl
diately after lyophilization The 0 acetyl
t of the other preparations was
mined after varying time of storage in
freeze-dried state Experiments indicate
loss of 0-acetyl and also of serological
y following storage of the lyophilized
n. As some deacetylation may take
during the extraction procedure the
indicate that within the intact bacte
ll all heptose molecules are acetylated
oheptoses have previously been found
ure only as constituents of the somatic
genic lipopolysaccharide from Gram-
ve bacteria D glycero-D galacto-
e is thus part of the specific polysaccha
f *Chromobacterium violaceum* (BN)

Except for the presence of heptose the
echaride antigen from strain L44 is
etely different from the 0 antigens of
negative bacteria The isolated antigen
ot contain lipid or hexosamine and has
low phosphorus content It is readily
e in water has a relatively low sedimen
coefficient and shows a very high
gical activity (work to be published)

li-labile 0 acetyl groups are present in
ell wall of both Gram positive and
negative bacteria (1) Some of the
ic 0-antigens of *Salmonella* contain 0-
(9 18) The best known 0 acetylated
rs are the extracellular hetero olysac
les—the "soluble specific su ces —
neumococcus (7) Accordin to *Rose*
(15) acetylated homopolysaccharides have
also been detected in microbial capsules

Small amounts of protein were invariably
found in all antigen preparations This may
indicate that protein is part of the macro-
molecular antigenic complex isolated The
trace amounts of other sugars than heptose
may represent contaminants

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12 *Partridge S M* Filter paper chromatography of sugars I General description and application to the qualitative analysis of sugars in

ıre solution approximately 50 per cent), 2 4 ml, ar (Difco), 15 g, distilled water ad 1000 ml The micals were *pro analysi* grade from Merck AG, rmstadt, Germany or Riedel de Haen AG, Han er, Germany The lactic acid was not less than 0 per cent after hydrolysis of anhydrides from H Chemicals, Ltd , Poole, England The ingre nts marked by asterisks were autoclaved separa and added aseptically after cooling to 60° C, rtly before pouring plates or distributing me um on tubes

3 *Complete media* As non synthetic media re referred to as complete media) were used (i) substrate designated L medium consisting of ptone (Difco), 10g Yeast Extract (Difco), 5g, Cl, 5g, distilled water ad 1000 ml, (ii) Heart usion Broth (Difco) (HIB), (iii) Nutrient oth (Difco) (NB) For typing plates was used 1 5 cent agar (Difco)

Buffers for pH variations The buffer solu s employed were either 0 1 M Sørensen s phos e buffer (used in the pH range 5 5 8 0) or tris made with 250 ml of an 0 2 M trishydroxy hylaminomethan solution (purriss Koch Light boratories Ltd Colnbrook, U K) per liter me m with varying volumes of 0 2 M HCL accord to the pH desired (used in the pH range 7 0 5 5) In e g the synthetic media the buffer sy n was added without adjustment in molarity ording to the phosphate contents already in the 9 or F media but the pH was always adjusted h the mono- or di basic phosphate as required r pH measurements was used the pH meter M 22 from Radiometer A/S Copenhagen Den rk with an electrode type GK 2021C

uantitation

For quantitation of the efficiencies of plating OP), the *agar layer method* (1) was used with per cent Agar (Difco) in the bottom layer (20) and 0 6 per cent in the top layer (5 ml) The cterial strains were in the late exponential wth phase One drop of the bacterial culture together with 0 1 ml of an appropriate ı to the top layer agar kept in a bath before plating For each combi hage and type of milieu triplicate made All incubations were made at overnight

atistical Evaluation

Adherence to the Poisson distribution for the antitation of *P aeruginosa* bacteriophages was ntrolled as reported in Table 1 For this purpose P determinations were made in tris buffered medium with pH adjusted to 7 0, and supple nts of 22 mM (0 4 per cent) glucose and 36 M $CaCl_2$ The subject of this study was phage 2

TABLE 1 *Compatibility of Observed Plaque Counts and the Poisson Distribution with Bacteriophage 2 in the Terminal Dilution Method*

Number of pla ques per plate	Observed frequency	Calculated frequency
0	81	76 2
1	99	104 4
2	66	71 5
3	35	32 7
4	9	11 2
5	6	2 7
6	1	0 6
7	3	0 1
8	0	0 0
Total	300	299 4

For experimental design see Materials and Me thods The compatibility of the observed frequen cies with the Poisson distribution was significant with a

$\chi^2 = 2\,492\ (\chi^2\ (3, \alpha = 0\,1) = 16\,3)$ (7)

The terminal dilution method was used, implying that the phage suspension was diluted such that mostly 0–2 plaques appeared on each plate The number of plates with from 0 to 8 plaques were then counted In Table 1, the observed frequency is compared with a calculated frequency for a Poisson distribution of the same mean as in the observed sample Standard statistical procedures were employed (10)

To distinguish plaque counts at the significance level of α = 5 per cent, a table wich allowed an immediate decision on the chance of two EOP's being different was employed (5)

RESULTS

Merits of Synthetic and Complete Media

The appearances of the typing reactions were compared on the synthetic lactate medium (SL) (9) and on the complete sub strates (L-medium and NB with 1 5 per cent Agar (Difco) and an enrichment of 36 mM $CaCl_2$) In my hands, mainly as a consequence of a more limited growth on SL, the typing reactions were more satisfactory on the complete media

Tested with tris buffer at the pH 7 0 and pH 8 5, the EOP's were higher with the L medium than with the synthetic substrates and slightly better than with unbuffered HIB The results

2 *Relative Efficiency of Plating (EOP) for 14* Pseudomonas aeruginosa *Bacteriophages with* *mologous Host Propagating Strains on Synthetic and Complete Media, pH Values 7 0 and 8 5, with or without Tris Buffer (T)*

Relative EOP § in	(Range)	Media*	pH	Buffer
3	(0–0 81)	F	8 5	T
3	(0–1 00)	F	7 0	T
9	(0 0 92)	L	8 5	T
J	(0 49–1 00)	L	7 0	T
1	(0 26–1 00)	HIB	7 0	–**
1	(0 27–1 00)	L	7 0	

relative EOP has been calculated within each phage as fractions of the highest EOP for that ge
text for the description of media
unbuffered

pical experiment made on 15 phages rded in Table 2 Similarly, compari e made in the L , M 9 , and F me ing phosphate buffer at the pH-va- 7 0 and 8 0, or tris buffer at 7 0, 8 5 Relative to the results on L agar, 's were (a) significantly inferior for ages tested on the M-9 medium and uced for 10 out of 15 phages on the m

-Optimum for Phage Plaque opment

l experiments were set up to investi- what degree with the buffer systems ated the pH was changed upon full bacterial growth Synthetic (F and M-9) and complete (L and HIB) liquid media of diffe rent pH values were employed Media were adjusted to pH 6 5 and 8 0 with phosphate buffer, and to pH 7 0 and 8 5 with tris buffer Readings of pH were made at the outset and after 24 hours incubation with vigorous aera tion at 37 °C

Duplicate tubes with 5 ml of the 16 different media resulting was inoculated with one of the propagating strains 7,44,68,119x, or Col 18

It turned out that at the pH values 6 5, 7 0, and 8 0, there was a change towards the alkaline in the HIB, L-, and F-media, least in F, whereas in the M-9-medium there was a

TABLE 3 *Changes in pH in Liquid Media upon 24 Hours Growth* of* 5 Pseudomonas aeruginosa *Strains*

Buffer	Mean change of pH (range) according to medium**			
	HIB	L	F	M 9
Phosphate	0 37 (0 3–0 5)	0 38 (0 3–0 5)	0 32 (2 5–0 6)	0 10 (0 to–0 2)
Tris	0 62 (0 5–0 8)	0 71 (0 6–0 8)	0 46 (0 4–0 5)	–0 80 (–0 4 to 0 2)
Phosphate	0 30 (0 1–0 4)	0 34 (0 2 -0 5)	0 15 (0 –0 2)	0 31 (0 4 to 0 1)
Tris	–0 10 (0 to–0 2)	–0 80 (0 to–0 1)	0 90 (0 –1)	–0 19 (–0 5 to 0)

erated cultures maintained at 37° C
our different media were employed the synthetic F and M 9 and the complete media L and HIB ecified in Materials and Methods The pH change is recorded as difference from uninoculated ntrol Duplicate tubes of each culture (medium and strain) were employed

TABLE 1 *Results of Diffusion In Gel Analysis of 5 M. avium and 20 M. intracellulare Strains*

Strain		Serotype (Schaefer)	Precipitinogenic factors demonstrated			
			b (γ)	c (α)	d	e
M. avium						
Faisan IV (MA4) Reference	(a)*		+	+	+	+
ATCC 15769	(b)	Avium 1	+	+		−
14141–1395	(b)	Avium 2		+		dev
Bačkovsky	(c)	Avium 2	+	+		+
Procházková	(c)	Avium 2	+	+	+	+
M. intracellulare						
14604–1610	(b)	Avium III	+	+	+	dev
13598 1079	(b)	Avium IV	+	+	dev	
Marsik	(c)	Avium IV	+	+	dev	
4443–1237	(b)	Avium V	+	+	+	
Mowray	(b)	Avium VII	+	+		
Formánek	(c)	Avium VII	+	+		
Melnik	(b)	Altman	+	+		
P 39	(b)	Boone	+	+	dev	
794	(d)	Boone		+		
9666/64	(b)	Darden	+	+		
C 3	(e)	Davis	+	+		
C 123	(e)	Davis	+	+		dev
C-210	(e)	Davis	+	+	dev	dev
C 272	(e)	Davis	+	+		dev
C 280	(e)	Davis	+	+		dev
C 291	(e)	Davis	+	+	dev	
ATCC 23434	(f)	Davis	+	+		
ATCC 23435	(f)	Davis	+	+		
ATCC 23436	(f)	Davis		+		
12305	(b)	Watson	+	+	dev	dev
S pek	(c)	Watson	+	+	dev	

+ identified by coalescence
dev identified by deviation
* Explanation of symbols for origin of strains
(a) Istituto Superiore di Sanitá Rome Italy Dr G Castelnuovo
(b) National Jewish Hospital Denver Colorado USA Dr W B Schaefer
(c) Tuberculosis Research Institute Prague Czechoslovakia Dr M Kubin
(d) Tuberculosis Research Institute Prague Czechoslovakia Dr Kovacs
(e) Department of Public Health Montgomery Alabama USA Dr D H Hosty
(f) State Veterinary Research Institute Brno Czechoslovakia Dr J Kazda

inogen c (α) was found in all strains The precipitinogen b (γ) was found in all strains except three which were of the serotypes Avium 2 Boone and Davis The precipitinogen d was demonstrated in ten strains of seven different serotypes In three strains (serotypes Avium 2 Avium III and Avium V) was the presence of the precipitinogen d indicated by coalescence In the remaining seven strains this precipitinogen was revealed by deviation of the line dD of the reference spectrum however, without coalescence

A line between the basin with the antigenic preparation under testing and the antiserum basin showing coalescence with the eE line of the reference spectrum was demonstr... three strains two of these b... type Avium 2 and the third strai... serotype Avium 1 (see Fig 2) ... nogen e was also shown by



Fig 3 Analysis of a strain serotype Boone (P 39) Factors b and c are demonstrable while the eE line of the reference spectrum is not influenced by the test strain material indicating lack of the corresponding precipitinogen e in the test strain

additional seven strains one belonging to serotype Avium 2, one to Avium III, four to type Davis and one to type Watson In the remaining strains e could not be revealed as exemplified in Fig 3

DISCUSSION

Agglutination procedures enable a subdivision of *M avium* and *M intracellulare* into several clearly distinguishable serotypes (*Schaefer* 1935, 1968, *Saito* & *Kubica* 1967) In the present study antigenic material obtained from agglutinogenically defined serotypes was compared with an earlier established *M avium* precipitation reference system (*Norlin*) Because this reference system originated from a culture filtrate and the tested antigenic preparations were obtained from disintegrated bacterial cells the experiments were oriented to determine primarily common interspecies crossreacting precipitinogens In addition the analyses comprised two precipitinogens previously found only in *M avium* and aviumlike strains (*Norlin* 1965)

The common mycobacterial precipitinogens γ and α were as could be expected from previous investigations registered in almost all of the strains examined in the present study The avium–aviumlike specific precipitinogen d was demonstrated in the reference strain and in one Avium 2 strain However, it could not be revealed in the remaining Avium 1 and Avium 2 strains The results concerning the precipitinogen d in the present study are not in accordane with previous investigations (*Norlin*) of the precipitinogenic patterns of *M avium* strains In those investigations d was revealed in the majority of the *M avium* strains analysed The difference in results might be explained either by a true absence of the precipitinogen d in the present *M avium* strains or by differences in the antigen preparative techniques It should also be noted that the precipitinogen d was found in nine out of the 20 *M intracellulare* strains belonging to various serotypes Thus the precipitinogen d should not be considered as an *M avium* specific precipitinogen

The presence or absence of the precipitinogen e in the strains tested is of some interest from the point of view of species differentiation This precipitinogen was demonstrated by coalescence in strains of the serotypes Avium 1 and Avium 2 (in three of four tested strains and in the reference strain) In addition it was revealed by deviation (definitely lower concentration than indication by coalescence) in one of the two serotype Watson strains and in four of the nine Davis strains The precipitinogen e was not however, found in strains belonging to the remaining serotypes (IV, V VII, Altman Boone Darden) Thus quantitative differences concerning the precipitinogen e indicate a possibility to differentiate *M avium* and *M intracellulare* into two different but closely related clusters However a conception of e as a precipitinogen specific for *M avium* can not be justified

It is obvious from the results presented that a more differentiated typing of the investigated strains was obtained by the agglutination technique than by the method of immunodiffusion A possible explanation for this difference could be the fact that by the analysis of agglutinogens surface antigens are revealed which carry determinants of more

pronounced specificity than endoantigens detected by precipitinogenic determinations. However, more penetrating analyses of precipitinogens employing a complete pattern might lead to a more advanced differentiation especially as it gives a more complete picture of the antigenic mosaic.

* This study was supported by grants from *WHO and Ellen, Walter and Lennart Hesselman's Foundation for Scientific Research*

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Acta path microbiol Scand Section B 79, 855—856, 1971

BRIEF REPORT

A SIMPLE TEST FOR PENICILLINASE PRODUCTION IN *STAPHYLOCOCCUS AUREUS*

I Ørstavik and *K Ødegaard*

Several tests, both quantitative as well as qualitative, have been devised in order to assess penicillinase production in strains of *Staphylococcus aureus* (7) *Gots* (1) incorporated in a solid medium a small amount of penicillin and a large inocolum of penicillin sensitive staphylococci The strain to be tested was then streaked on the surface Penicillinase producing strains destroyed the drug in its vicinity and allowed the sensitive staphylococci to grow like satellites around the streak Several modifications of this technique have been introduced (4 5) *El Ghoroury* (2) made a uniform inoculation of a plate with a sensitive strain. Filter paper discs containing penicillin were placed on the surface and a loopful of a broth culture of the strain to be tested was added A restriction of the inhibition zone was observed in penicillinase producing strains when compared with penicillin sensitive strains or with the inhibition zone around the penicillin containing paper disc without any bacteria added

The present report describes a simple qualitative test for penicillinase production in *Staph aureus* based on similar principles

Method

Plates with a diameter of 14 cm containing Mueller Hinton medium (Bacto, Difco) were seeded by flooding with a 3 ml suspension of a penicillin sensitive strain of *Staph aureus* (P209/Oxford) diluted so as to give a dense but not confluent growth of colonies (3) Surplus inoculum was removed by a pipette and the plates allowed to dry for one hour at room temperature A solid streak of the strain to be tested was then made on the surface of the plate, and a filter paper disc containing 10 µg penicillin G (AB Biodisk, Stockholm, Sweden) was placed in the middle of the streak Following half an hour of prediffusion at room temperature, the plates were incubated at 36–37° C and read 18–20 hours later

Explanation of results Strains without penicillinase production had no effect on the inhibition zones around the penicillin G discs all zones being circular and of about the same size The inhibition zones below streaks of bacteria presumably producing penicillinase, however, were of reduced size and not circular In the periphery of the penicillin diffusion zone next to the bacterial streak the penicillin was destroyed to such a degree that colonies of the sensitive test strain could be formed The inhibition zones under strains producing penicillinase thus showed a coffee bean like appearance (Fig 1)

Some strains showed antibacterial activity against the test strain, presumably due to staphylococcin production, but this did not obscure the distinction between penicillinase producing and non producing strains (Fig 2)

Testing of the method The method was tested on 216 coagulase producing strains of *Staph aureus* isolated from various clinical specimens during a period of four weeks in May 1971 The strains were all subject to the routine test used in the laboratory for antibiotic sensitivity by agar diffusion, using the paper disc method (AB Biodisk)

Results

The outcome of the tests for penicillinase production was compared with the diameters of the inhibition zones for the penicillin G discs (10 µg) in the routine sensitivity test (Table 1)

The strains may be divided into two major groups according to the inhibition zone caused by penicillin G in the routine test. Strains with zone diameter less than 25 mm all gave coffee bean like zones in the penicillinase test and were grouped as penicillinase producers Penicillinase was not detected in strains with zones greater than 34 mm

Received 20 x 71 from the *National Institute of Public Health Department of Bacteriology, Oslo Norway*

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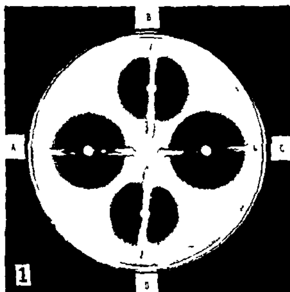


Fig 1 Test for penicillinase production The medium has been seeded with the test strain of *Staph aureus* (P209) The four strains to be tested were streaked at A, B C and D and discs containing 10 μg penicillin G placed in the middle of the streaks Strains at B and D produce penicillinase, strains at A and C do not

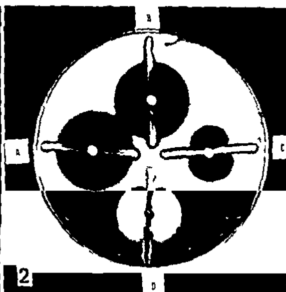


Fig 2 As in Fig 1, but strains at A, B and C show antibacterial activity, presumably due to staphylococcin production Strains at C and D produce penicillinase, strains at A and B do not

TABLE 1 *Penicillinase Production and Penicillin G Sensitivity in 216 Strains of Staph aureus*

Test for penicillinase production	Zone diameter in routine sensitivity test			
	<25 mm	25 34 mm	>34 mm	Total
Positive	99	2	0	101
Negative	0	1	114	115
Total no *of strains*	99	3	114	216

Only three strains showed inhibition zone diameters in the intermediate range of 25 34 mm and two of these strains were found to produce penicillinase by the described test

The strains were also tested for antibiotic sensitivity to methicillin with discs containing 30 μg of the drug According to the manufacturer penicillinase production is indicated if the methicillin zone is considerably larger than the penicillin G zone (6) Of the 101 strains found to be penicillinase producers with the present method 99 had larger methicillin than penicillin G zones One strain showed zones of equal size and one showed larger penicillin zone All the 115 penicillinase negative strains showed larger penicillin than methicillin zones in the routine agar diffusion test for antibiotic sensitivity

Conclusion

The described method for penicillinase detection correlates well with two routine checks on penicillinase production by *Staph aureus* Size of zone inhibition by the penicillin G disc and comparison between this zone size and that produced by the methicillin disc.

The test is easy to perform and 4–6 strains may well be examined on one plate Previously described methods for penicillinase detection have required media containing penicillin (1, 4, 5) or broth cultures of the organisms to be tested (2) or special discs and reagents (6) The present method only requires equipment at hand in the laboratory for routine use and the strains to be tested may be picked from colonies on solid media

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